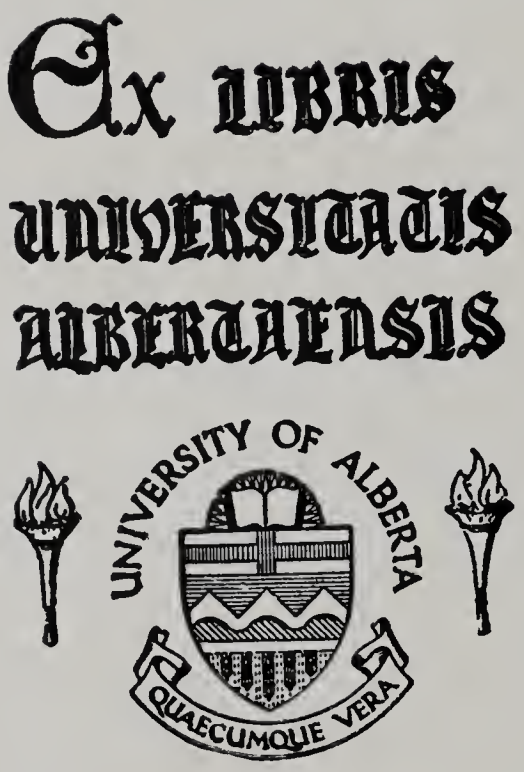

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GAS CHROMATOGRAPHY OF INDOLE AUXINS

by

WALTER DEDIO

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Gas Chromatography of Indole Auxins" submitted by Walter Dedio in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The gas chromatographic technique was investigated as a possible method for separating and estimating indole auxins from plant extracts.

Most efficient separations of indole auxins were obtained with silicone gums, SE-30 and SE-52. Other substrates producing less satisfactory results included neopentyl glycol succinate and QF-1 (trifluoro propyl methyl silicone). The optimum operating temperature was found to be 205° C. Conditioning of the column was found necessary.

For effective separation by gas chromatography, it was necessary first of all to fractionate the indoles into acidic and neutral groups. Neutral indole compounds were gas chromatographed directly, while the indole acids were made more volatile by esterifying them either with BF_3-methanol or diazomethane. Tryptamine chromatographed either as the amine or as an acetyl derivative. Tryptophan chromatographed as a methyl ester. Quantitative conversion of indole aldehydes into more stable acetals for gas chromatography was not successful.

A neutral indole compound was isolated from cabbage by this technique but its identity was not established since its retention time was longer on the SE-52 column than any of the neutral indoles studied. The spectrophotometer was used to confirm its indolic nature.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

TABLE OF CONTENTS (Con't.)

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Indole auxins have been detected and estimated either chemically or biologically. Rather large amounts of plant material are required for chemical methods, but these are more specific than bioassays. Ordinarily the indole compounds are isolated by extraction of the tissue with an appropriate solvent and subsequently separated by paper chromatography. Quantitative estimation can be carried out either with a bioassay or by spectrophotometric methods. The bioassay can be employed only for the physiologically active indoles, while the spectrophotometric techniques are not specific.

Gas chromatography has in the last few years been extended to a large number of compounds, including aromatics and steroids. The application of this technique to indole auxins has been reported from only one laboratory (80, 92).

Further investigation of the application of gas chromatography to indole auxins seemed worthwhile since in theory, at least, it should provide a method of separation and quantitation of the indoles contained in plant extracts. In addition it should enable the collection of individual compounds for further characterization. Moreover, it could be used to check on the purity and to purify supposedly authentic indole compounds. Therefore, the work reported in this thesis was intended to extend the application of this technique to a series of indole compounds. The major portion of the work was devoted to finding suitable column substrates and to the preparation of various derivatives of certain indoles. The remainder of the thesis was concerned with a comparison of the efficiency of various columns and the application of these procedures to plant extracts.

LITERATURE REVIEW

I. Extraction and Detection of Indole Auxins

(a) Extraction

Extraction procedures for indole auxins have been described in detail by Larsen (61) and Leopold (62). The earliest method of extracting auxin was by diffusion into agar blocks. Went (106) in 1929 and Avery et al. (4) in 1937, reported that certain plant organs secrete auxin into plain agar. The diffusion method, however, gives no information as to the amount of active material in the plant tissue at a given moment and furthermore some enzymatic inactivation of auxin is known to take place at the cut surface.

More complete extraction of auxins has been obtained with certain solvents. Water has been used to a limited extent because some conversion or destruction of indole auxins occurs in aqueous solutions. Extraction with diethyl ether has been most widely employed. The procedure has been described by Van Overbeek et al. (104) and Bonde (13). Some inactivation of auxins occurs but this can be prevented by working at a temperature of 1^{o} C. Alcohol is favored by some workers because it results in more complete extraction of auxins. Linser (63) pointed out that alcohol penetrates the tissues more efficiently than ether and prevents the formation of foam which occurs in ether extractions, but it has the disadvantage of being more difficult to remove than ether.

(b) Fractionation of Extracts

The simplest procedure for fractionating auxins into acidic and neutral groups is to partition between appropriate solvents. A common system is ether and an aqueous solution. By adding $NaHCO_3$ to water, the acid fraction can be extracted (55) into the aqueous solution. Powell (80), however, preferred to use methylene chloride instead of ether.

Separations of indole compounds have been made with column chromatographs. Linser (64), used aluminum oxide for packing. The ethanol extract was poured through and the different bands which were detected with an ultraviolet lamp were eluted with sodium hydroxide. Fisher and Behrens (30) described the separation of indole derivatives by partitioning on a column of cellulose powder. More recently, Powell (79) developed a simple and rapid method employing silica gel columns hydrated with 0.5 M formic acid. The solvents used were petroleum ether and butanol with the proportion of the latter being increased stepwise as the more polar derivatives were eluted.

The method most widely used for separation and identification of plant growth substances is paper chromatography. This has been reviewed by several workers (7, 61, 62). It is customary to run control chromatograms with synthetic compounds as markers. The majority of the workers apply the extract either in ether or alcohol and run it under alkaline conditions (isopropanol : ammonia : water). This solvent system gives good separation of acidic indoles, while

the neutral ones tend to be bunched close to the solvent front. Some workers (74, 93) have criticized the use of ammonia as it breaks down the more labile derivatives. Water alone will separate indole acetic acid, indole butyric acid, indole acetonitrile and ethyl indole acetate. Nitsch recommends a mixture of hexane and water for separation of neutral auxins. An apparatus has been described for carrying out paper chromatography and paper electrophoresis simultaneously (5), but this technique has not been used extensively.

A variety of chemical reagents can be employed to detect indole derivatives on paper chromatograms. The most widely used are the Salkowski and Ehrlich reagents. The Salkowski reagent consists of $HClO_4$ with some $FeCl_3$, while the Ehrlich reagent consists of p-dimethylaminobenzaldehyde in HCl. Other reagents that have been used to a lesser extent are KNO_2 and HNO_3 in ethanol and cinnamaldehyde in ethanol and HCl. Recently a more sensitive chromogenic agent, p-dimethylaminocinnamaldehyde (42) has been described.

(c) Estimation of auxins

The chromogenic agents may be used to develop color in a solution and the amount of auxin can be estimated colorimetrically. The Salkowski reaction is highly specific for indole acetic acid. The reaction involves $FeCl_3$ and a mineral acid such as sulfuric acid (Tang and Bonner (97)) or $HClO_4$ (Gordon and Weber (35)). The Adamkiewicz reaction with glyoxylic acid and sulfuric acid has been used, but is not reliable for IAA as it is more specific for other indole derivatives. The nitroso-indole reaction (sodium nitrite and

nitric acid) is specific for indole, the color intensity being three times that of IAA. Algéus (1) has employed the Ehrlich's reaction for determining IAA.

Fletcher and Zalik (31) preferred to determine IAA quantitatively by ultra-violet absorption. This method, however, can be used only with relatively pure extracts as any foreign substances will greatly interfere with the determination. Stowe and Schilke (92) have been working with spectrophotofluorometric methods in estimating various synthetic indoles and have claimed sensitivity to a one nanogram (10^{-9} g.) level. This method like the ultra-violet absorption method is sensitive to interfering substances and pre-purification is necessary before indole auxins can be estimated in plants. Both spectrophotometric methods are not specific for various indoles and it is therefore necessary that only one indole derivative be present. Maximum ultraviolet absorption occurs at about 280 - 285 mμ and the major fluorescence peaks are between 360 and 375 mμ.

Stowe has been working with gas chromatography as a prepurification step for estimation by the spectrophotofluorometric method. As the application of gas chromatography in the separation and estimation of indole auxins is the subject of this thesis, it will be discussed in a separate section.

(d) Bioassay

Bioassay of auxins has been reviewed by several workers (7, 61, 62). The most widely used plant for bioassay is the Avena. In the Avena curvature test, an agar block containing the auxin is placed on one side of the coleoptile tip. After 90 minutes, the angle which the straight base of the coleoptile makes with the tangent to the curved portion at its extreme end is measured. Curvatures are recorded on bromide paper placed closely behind the plants and parallel to the plane of curvature. A shadow print is obtained and the angle of curvature is measured from it. The physiological basis of this test is that the transport of auxin is polar (downward) and the resultant elongation causes a curvature away from the side to which the auxin has been applied. The disadvantage of this test is that it is rather difficult to perform.

Nitsch (74) has developed a simpler test in which the first internode of Avena is used. The physiological basis for the first internode test is that the auxin diffuses uniformly throughout the internode and this is enhanced by rotating the coleoptiles either by hand or on a wheel. The internode test has the advantage of not showing response to amino acids and is not sensitive to inhibitors.

The slit-pea curvature test has been mostly used in the study of synthetic auxins, as large amounts are required. The test is based on measurement of auxin-induced curvature which is produced by tissue tension in the longitudinally slit internodes of etiolated pea seedlings. Polar transport is not involved in this test.

The fact that young roots are more sensitive to auxins than young stems has been exploited in root tests. At low concentrations of auxin root growth is promoted, while at higher concentrations inhibition occurs. However, other factors such as organic acids and pH will affect the results. This makes variability high in root tests, and for this reason, they are not used extensively.

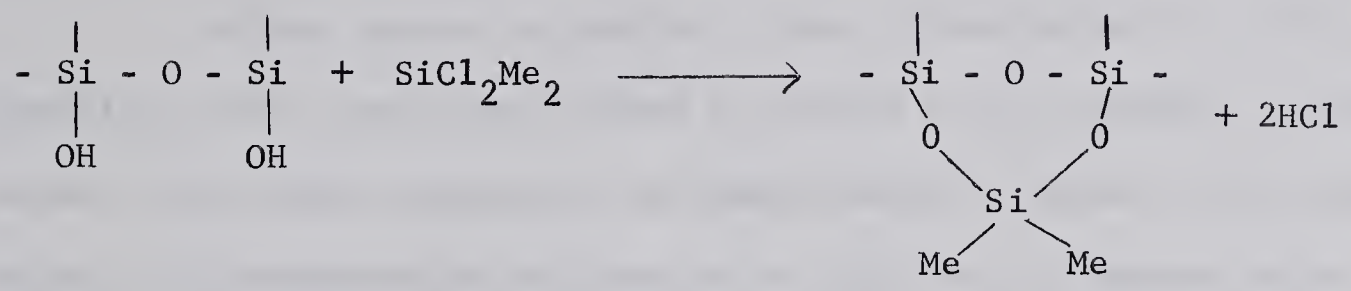
II. Gas Chromatography of Indole Auxins and Related Compounds

Introduction

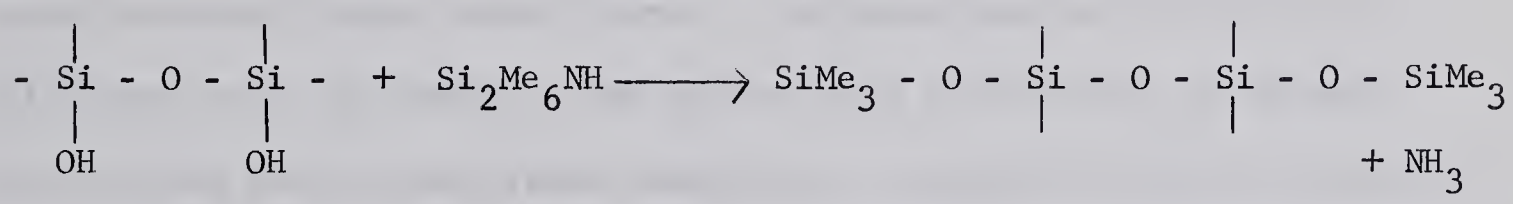
Gas chromatography was first introduced as an analytical technique in the separation of fatty acids by James and Martin in 1951 (51). Since then the technique has been investigated for application to other classes of compounds. Probably the greatest improvement in the technique has been the introduction of highly sensitive detectors such as the argon and hydrogen flame ionization gauges, which can detect as little as one nanogram of a substance.

Liquid phases, varying in their degree of polarity have been introduced. Among the silicones, SE-30 (silicone gum with methyl side chains) is a non-polar phase which has been used as a thin coating (< 5%) in the separation of steroids (103). Silicone gums have an advantage in that they are stable at high temperature and are rather inert to most groups of compounds. Their polarity may be increased by introducing certain side chain groups such as phenyl in SE-52, which is a semi-polar substrate and fluorine in QF-1, a polar substrate.

Prior to 1960, firebrick had been used as a solid support but since then it has been replaced by calcined diatomaceous earths sold under trade names such as Anakrom, Chromosorb and Gas Chrom. Solid supports are made more inert by treatment with silane (12, 48). Dimethyldichlorosilane reacts with hydroxyl groups located on the silica surface in the following way (12).



Two adjacent hydroxyl groups are required for the reaction to proceed and for this reason another substance, hexamethyldisilazane has been widely used. It reacts quantitatively with hydroxyl groups according to the reaction.



As the reaction of substances with the solid support is reduced by these treatments, there is a tendency to use thin films of liquid phase. This enables one to work at lower temperatures (103). However, quantitative analysis of compounds with polar functional groups remains a problem. Horning et al. (47) have emphasized the fact that polar groups within the steriod can cause substantial loss due to partial adsorption to the support. This problem may be minimized by preparing derivatives in order to protect the polar groups and in some cases a heavy coating of the liquid phase will mask the adsorptive sites.

Several reviews on biochemical applications of gas chromatography have been written recently (18, 19, 49, 66, 96). In these the indole auxins are included under (a) aromatic amines, (b) aromatic aldehydes, (c) aromatic acids, and (d) amino acids.

(a) Aromatic amines

Amines present a problem in gas chromatography in that they contain a polar group which tends to adsorb to the packing. Several workers have done separations of sympathomimetic amines. Fales and Pisano (25) demonstrated separation of high boiling amines which included serotonin, histamine and tryptamine with a 4% SE-30 packing on Gas Chrom. P. Parker et al. (76) developed a method of screening sympathomimetic amines and related classes by gas chromatography. Several other workers have experimented with amine derivatives in order to obtain more stable forms. Brochmann-Hanssen and Svendsen (15) were able to separate some amines more efficiently as acetone derivatives and as acetylated compounds. Extensive study of biological amines has been done by Horning et al. (17, 46, 102). Conditions were described for the catecholamines and other amines through the use of acetylated derivatives. A two-component mixed stationary phase of moderate polarity, comprised of 7% F-60 silicone oil with 1% Polymer EGSS-Z was employed. Amines were acetylated using acetic anhydride with pyridine as a catalyst. Certain derivatives were best separated with a 10% neopentyl glycol succinate on 100/200 Gas Chrom. P packing. Landault and Guichon (57) have recommended Teflon as the solid support.

(b) Aldehydes

Gas chromatography of aldehydes has been dealt with in reviews by Horning and Vanden Heuvel (49) and Burchfield and Storrs (18). The more volatile aldehydes can be chromatographed directly with non-polar columns such as the silicones. However, with aldehydes which have higher boiling points, some loss occurs because of the high activity of the aldehyde group.

In 1960, Gray (36) prepared dimethyl acetal derivatives of fatty aldehydes and separated them on an Apiezon column. They were prepared by refluxing the aldehydes in anhydrous methanol with hydrochloric acid. More recently, other derivatives were experimented with. Farquhar (26) outlined two methods by which methyl esters could be prepared from aldehydes. One consisted of hydrolysis of dimethyl-acetals and subsequent oxidation to acids with silver nitrate. The acids were then methylated with HCl-MeOH. In the other method, the dimethylacetals were reduced to alcohols with lithium aluminum hydride and the alcohols subsequently acetylated with acetic anhydride. The methyl esters of aldehydes were also employed by other workers (37, 75, 85).

Soukup et al. (87) separated carbonyl compounds including aromatic aldehydes as 2,4-dinitrophenylhydrazone derivatives using a silicone substrate, SF-96.

(c) Amino Acids

Amino acids having two polar functional groups, the amino and carboxylic group, are not themselves volatile enough to be gas chromatographed directly. However, several types of derivatives have been prepared which are more volatile.

The earliest derivatives were the aldehydes prepared by reacting amino acids with ninhydrin resulting in one less carbon. Bier and Teitelbaum (9) used a silicone column which separated the aldehydes of alanine, 2-aminobutyric acid, valine and leucine. Bayer (6) was able to convert some amino acids into esters by treating with methanol-HCl. The esters were extracted with ether after alkalinization of the reaction mixture and chromatographed on a silicone high-vacuum grease. Hydrochloride salts of the amino acid esters were injected directly into the chromatograph by Saroff et al. (73, 84) using a polyethylene glycol adipate and 2% neopentyl glycol succinate column. However, histidine, tyrosine and tryptophan gave no peaks. Furthermore, some of the esters were lost because free bases tended to form diketopiperazines.

More effort has been directed to N-acyl amino acid esters than other derivatives. After Weygand (107) reported the volatility of N-trifluoroacetyl esters, Bayer (6) separated esters of glycine, valine, leucine and proline. Youngs (114) elected to prepare N-acetyl-n-butyl esters with n-butanol saturated with anhydrous hydrogen chloride. The reagents were then removed to dryness by vacuum distillation and the residue treated with acetic anhydride.

After removal of acetic anhydride the acyl esters were chromatographed with a 25% hydrogenated safflower oil. Weygand (108) has modified this procedure and was able to chromatograph more amino acids. After the esterification procedure with methanol-HCl, the residue was combined with methanol, triethylamine and methyltrifluoroacetate. The solvent was evaporated and the derivatives were extracted into ethyl acetate from an aqueous solution. Wagner and Winkler (105) reversed the esterification and trifluoroacetylation steps. Trifluoroacetic acid and trifluoroacetic anhydride were added first. After completion of the reaction, the solution was evaporated and the residue was taken up in methyl acetate and esterified with an ethereal solution of diazomethane. The analysis was done with an Apiezon L and sodium caproate column. Johnson, Scott and Meister (52) did extensive studies with N-acetylamino acid n-amyl esters. Hydrogen bromide was used instead of hydrogen chloride and the derivatives were separated with a polyethylene glycol column.

All the above methods of preparing n-acyl amino acid esters destroyed the labile amino acids such as tryptophan. Zomzely, Marco and Emery (115) employed a mild esterification procedure in preparing N-trifluoro-acetyl-n-butyl esters of such troublesome amino acids as threonine, serine, lysine, arginine, tyrosine, histidine, tryptophan and cystine. The procedure included the addition of dimethylformamide and dibutoxypropane during esterification which was carried out at 55 - 60^{o} C for 3 hours. A neopentyl glycol succinate column was used. Cruickshank and Sheenan (23) used dimethyl sulfite in addition to methanol and hydrochloric acid, while Makisumi and Saroff (68) found

diacetyl derivatives of tryptophan are more stable and chromatographed the best. The first quantitation of amino acids was done by Hagen and Black (41) who found a direct relationship between peak areas and the amount of amino acid derivatives.

Amino acid esters (10) and N-acylamino acids (45) react with trimethylchlorosilane to form N-trimethylsilylamino acid esters and N-acyltrimethylsilyl esters. Improvements in the procedure (82) in preparation of silyl derivatives have resulted in 87 - 97% yields and involves the reaction of free amino acids with a slight excess of N-trimethylsilyldiethylamine.

Some amino acids can be chromatographed as phenylthiohydantoins (78) on an SE-30 column. Most of the N-2,4-dinitrophenyl derivatives of the amino acids may also be determined by gas chromatography after their conversion to methyl esters with diazomethane (78). Even the unstable N-2,4-dinitrophenyltyrosine and tryptophan can be chromatographed on a 2% SE-30 column.

(d) Aromatic acids

There has been much interest in the separation of aromatic acids that are found in urine. These acids are themselves not volatile enough to be chromatographed and it is necessary to prepare volatile derivatives. Methylated derivatives have been prepared using methanolic HCl or BF_3-methanol (71), but a difficulty that was encountered was the appearance of brown pigments. A more suitable method was the reaction of the acids with an ethereal solution of diazomethane at room temperature. Diazomethane can be prepared by

reaction of KOH with N-methyl-N'-nitro-N'-nitroso-guanidine (70) or p-tolylsulfonylmethyl nitrosamide (24) and distilling it off with diethyl ether. The advantages of the method are that more complete methylation is obtained and the reagents, ether and diazomethane, are evaporated off easily.

Williams (111) has described several methods for esterifying different acids. The simplest procedure involves simply adding diazomethane in ether in excess and then evaporating the reagents. Acids containing an hydroxy group can be methyl esterified to O-methyl ether by reacting the acid overnight with diazomethane in a mixture of diethyl ether and methanol in a capped vessel. In order to avoid a common derivative a third method has been devised (112) which produces methyl esterified, O-acetoxy compounds. The acids are first acetylated with acetic anhydride, with trifluoroacetic acid as a catalyst and following evaporation, are esterified with ethereal diazomethane. Another procedure, not extensively studied is the preparation of O-trimethyl-silyl methyl ethers. In 1958, Langer, Pantages, and Wender described this procedure for a variety of phenols (58).

Indole auxins

Gas chromatography of indole auxins has so far been reported only by Stowe and Schilke (92) and Powell (80). Indole auxins are difficult to chromatograph in that they contain various functional groups and it is difficult to select a packing that will not react with one or more of these. They (80, 92) reported best results with columns of Versamid 900 and QF-1. Other packings which gave partial

success were polyneopentyl glycol succinate, polyethylene glycol isophthalate and poly-m-phenyl ether.

The indole compounds were fractionated into acidic and non-acidic groups. The acidic indoles were esterified with BF_3-methanol or diazomethane. The latter procedure gave a more complete esterification. The esters were satisfactorily separated on Versamid 900, polyneopentyl glycol succinate and QF-1.

The neutral and basic indoles were chromatographed directly and the best results were obtained on a Versamid 900 column, even for the labile compounds such as tryptamine and the aldehydes. No response was obtained from indole acetaldehyde on a QF-1 column, while tryptamine decomposed this substrate.

Clearly, there are still some problems to be solved before this technique can be used routinely in separating auxins from plants and estimating them. One problem is that of obtaining an extract that is free from interfering substances. This may be done by column chromatography or partition fractionation prior to injection into the gas chromatograph. Another problem is that of sensitivity. The amount of natural auxins present in plants is too low for detection by gas chromatography and other methods such as spectrophotometry or bioassay may have to accompany it.

Collection of Eluates

Ordinarily, the eluates are collected with Teflon tubing. Condensation occurs in a loop and this is rinsed out. Another method

of collection is with a plug of cotton or glass wool soaked in a solvent and placed in a glass tube.

These methods are satisfactory for large amounts as complete condensation occurs. However, as the quantities are decreased, there is tendency for formation of aerosol particles which cannot be condensed. A number of methods have been reported in which the aerosol particles are trapped. Such devices may depend on the maintenance of a temperature differential between a heated inner wall and cooled outer wall (88, 98) or on the use of a cold trap and an electrostatic precipitator (100). A procedure for condensing argon, a carrier gas along with the sample in a liquid nitrogen trap has been described (95). Hornstein and Crowe (50) improved this method by using CO_2 as a carrier gas as this gas has a much higher boiling point than argon.

III. Biosynthesis and Metabolism of IAA

(a) Biosynthesis

Although many indole compounds are known to occur in plants, the biosynthetic pathway of IAA* has still not been completely elucidated. IAA has been reported in more than 20 species of plants

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* The following abbreviations will be used; IAA, indoleacetic acid; IPA, indolepropionic acid; IBA, indolebutyric acid; IPyA, indolepyruvic acid; IAN, indoleacetonitrile; IAAld, indoleacetaldehyde; IAld, indolealdehyde; ICA, indolecarboxylic acid; IAM, indoleacetamide.

and in almost all organs. Other acidic indole auxins that have been isolated are IPA (11, 29), IBA (65), and IPyA (65, 83).

Among the neutral indole auxins, IAN has been confirmed to be present in cabbage by Jones (44) and in other plants. Other indole compounds that have been isolated are IAAld, IAld and tryptamine. Tryptophan has been definitely shown to occur in plants. Furthermore, it has been known that tryptophan content varies with the stage of plant growth (101). At flowering time, the level is higher and this enables more production of IAA. Tryptophan, therefore, long occupied a central position in indole auxin biogenesis.

Thimann (99) found that the amount of IAA produced by Rhizopus would depend on the amount of tryptophan present in the medium. Boysen-Jensen (14) observed that other amino acids can be substituted for tryptophan. These amino acids are presumably converted to tryptophan prior to synthesis of IAA. In higher plants, conversion of tryptophan has been demonstrated in live tissues of spinach, pineapple, pea and tomato, tomato shoots and other organs of various plants. The tryptophan synthetase system which couples serine and indole, has been reported by Greenberg and Galston (39) in pea seedlings and by Mudd and Zalik (72) in the tomato.

There seem to be several pathways from tryptophan to IAA. IAAld appears to be the immediate precursor. Larsen (59) found that IAAld was readily converted to IAA in excised coleoptiles and juice of coleoptiles. Working with pineapple, Gordon and Nieva (34) have demonstrated the presence of an enzyme system capable of this conversion.

Larsen (60) reported that two moles of naphthalene acetaldehyde yielded one mole of naphthaleneacetic acid and it is thought that a similar stoichemical relationship holds for the synthesis of IAA. Two moles of IAAld will yield a mole of IAA and a mole of tryptophol. Gordon and Nieva also found that IAAld was formed from tryptophan via tryptamine. This observation has been reported in various plants, fungi (22) and bacteria (77). Clarke and Mann (20) were able to obtain a purified amine oxidase from pea seedlings which gave high yields of IAAld from tryptamine.

Tryptophan also can be converted to IAAld via IPyA. Several workers have reported the presence of IPyA in plants, but this has been questioned because ammonia was used in the chromatography and this has been known to destroy IPyA (8). However, with improved techniques more evidence has accumulated indicating that this compound may be an intermediate (83). Enzyme preparations from spinach were shown to be able to convert IPyA to auxin but incapable of converting tryptamine (110). An aqueous solution of IPyA decomposes to IAAld (89) upon standing.

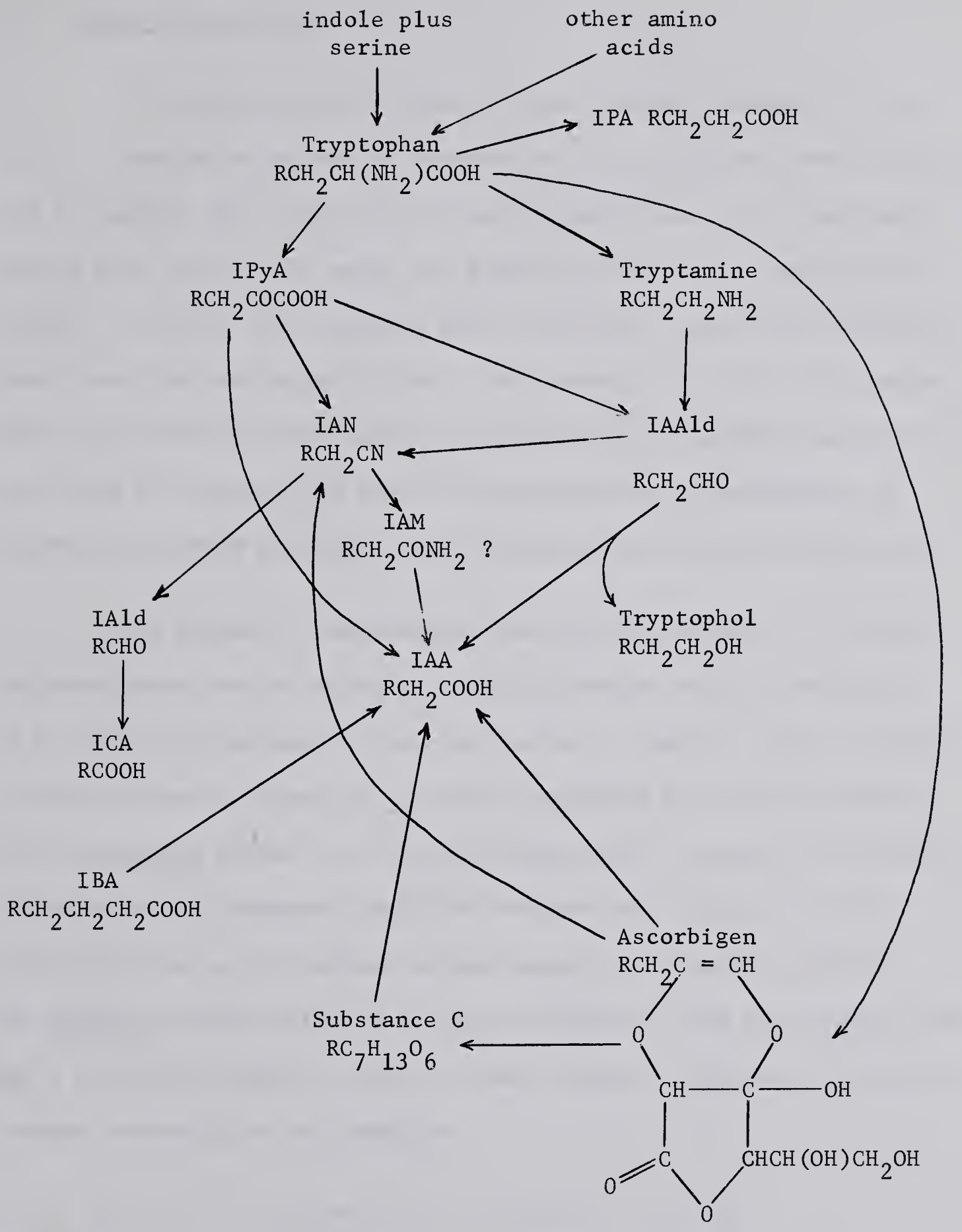
Another indole compound present in abundance in some species which may be an important intermediate is IAN. It has been generally accepted that it is present in the cabbage and has also been found in oats and watermelons. There is still much confusion as to how IAN originates. When radioactive tryptophan was fed to Savoy cabbage (56), IAN was produced. Other radioactive products formed were IPyA, IAA, IAld and ICA. It is very probable that IAN is the immediate precursor

of IAA. Also, since it has been found that IAN undergoes α-oxidation, it is most likely that radioactive IAN is also the precursor of radioactive IAld and ICA (27). It has been suggested that IAN is hydrolysed to IAA but a number of workers were not able to isolate the expected intermediate, IAM. Two more biosynthetic pathways have been suggested, but good evidence has not been obtained for either of them. One involves the conversion of IAAld to IAN via indole acetaldoxime (67) and in the other IPyA goes via its oxime to IAN (90).

IBA undergoes β-oxidation to IAA in certain plants (3, 28) and IBA itself acts as an auxin. In microorganisms, tryptophan can be de-aminated to IPA (21) and this compound also has some auxin activity.

Recently some Czechoslovakian workers have reported on the importance of ascorbigen in synthesis of IAA. When ascorbigen was chromatographed in ammoniacal solvents, it was found that some of it would break down to IAN and IAA. This has been supported by Söding (86) who found that ascorbic acid was able to increase growth. Ascorbigen content parallels that of IAA and is metabolized rapidly at the time of growth.

Radioactive ascorbigen was obtained by Kutáček (56) when tryptophan-3-C^{14} was fed to Savoy cabbage. Ascorbigen can then be converted directly to IAA or first to IAN. The latter reaction occurs in ammoniacal solutions as in paper chromatography. In alkaline solutions, ascorbigen loses a carbonyl group and is converted to a "substance C" and this compound in turn yields IAA. The interconversions of indole compounds in plants can be summarized by the following scheme.



Other routes are possible, but they are not definitely established. Two other indole intermediates, indole glyoxylic acid (109) and indole glycollic acid (40) are not included as their position is not clear.

(b) Destruction of IAA

The inactivation of IAA has been reviewed recently by Ray (81). A definite pathway of degradation of IAA has not been established and it appears that more than one may be operating. It is generally agreed that IAld is the major end product and some intermediates are present. Fischer (29) reported that ultraviolet light and riboflavin sensitized photooxidation of IAA. The products were IAld and another substance identical with indole-3-glycolic acid. Another factor, Mn^{++} was found to increase the rate of IAA oxidation in horseradish by Kenten (54) while at higher concentrations oxidation was inhibited.

In enzymatic degradation, Manning and Galston (69) found two substances, one of which is formylkynurenine which is produced as a result of breakage of the ring nucleus. However, this is doubted by other workers. Stowe et al. (91) suggested the product produced by the Omphalia enzyme to be 3-methyldioxindole. However, in further experiments this compound could not be isolated. There is still uncertainty as to the pathway in the enzymatic formation of IAld. The Omphalia enzyme oxidized indole-3-isobutyric acid more rapidly than IAA (94) and it appears that it is not oxidized stepwise as has been thought according to the equation.

$$ICH_2COOH \longrightarrow ICHOH \cdot COOH \longrightarrow ICOCOOH \longrightarrow ICHO$$

The oxidation of indole-3-isobutyric acid cannot occur this way because of the 2 methyl groups. Stutz (94) discovered that with Lupinus enzyme supplied with cytochrome C, both IAA and indole glycolic acid were oxidized to IAld.

Other products besides IAld have been reported as a result of IAA destruction. Ethylindoleacetate has been detected by many workers in various seeds and may be the result of IAA inactivation. Andreae et al. (2) have discovered that IAA conjugates with aspartate to produce indole-acetyl-N'-aspartic acid in certain plant tissues. In grasses, indoleacetamide has been observed to be the conjugate (33).

A most widely accepted hypothesis of IAA destruction is the flavoprotein-peroxidase hypothesis. IAA oxidation is believed to be mediated by a coupling system involving IAA oxidases, which consists of a peroxidase, since H_2O_2 is required, and a flavin enzyme forming H_2O_2 from O_2. This was proposed by Galston, Bonner and Baker (32) and can be formulated as follows:

$$IAA + H_2O_2 \xrightarrow{\text{peroxidase}} P_1 + 2H_2O$$

$$P_1 + O_2 \xrightarrow{\text{flavoprotein}} P_2 + H_2O_2$$

MATERIALS AND METHODS

Equipment

The gas chromatograph equipment used in this study were the Aerograph Model A-90-S and the "Hy-fi" Model A-600-B. The A-90-S model was equipped with a thermal conductivity cell detector and supplied with a 250 milliampere current. Stainless steel columns, 1/4 inch in diameter and varying in lengths from two and one-half to ten feet were used. Helium was used as the carrier gas and the flow rate was adjusted from 40 to 90 cc per minute depending on the type of column packing. The flow rate was measured with a gas burette and soap film.

The "Hy-fi" Model A-600-B was equipped with a hydrogen flame ionization detector. Five and nine foot stainless steel columns, 1/8 inch in diameter were used. Nitrogen was the carrier gas and the flow rate was about 10 cc per minute. Hydrogen for the detector was produced by a Model A-650 hydrogen generator.

Solid Supports and Liquid Substrates

Various liquid phases were tried including SE-52, neopentyl glycol succinate, QF-1, Versamid 900 and a combination of Versamid 900 and SE-52. They were coated on Chromosorb W, 60/80 mesh, HMDS or Anakrom ABS, 70/80 mesh. Table I lists the trade names, composition and suppliers of the solid supports and liquid substrates used in gas chromatography of indole compounds.

Table I. Trade Name, Composition and Supplier of Solid Supports and Liquid Substrates Used in Gas Chromatography of Indole Compounds

Trade Name	Composition or Description	Source
SE-30	Methyl silicone	Wilkens Instrument and Research, Inc.
SE-52	Methyl phenyl silicone	Wilkens Instrument and Research, Inc.
Neopentyl glycol succinate		Applied Science Laboratories, Ltd.
QF-1	Trifluoro propyl methyl silicone	Applied Science Laboratories, Ltd.
Versamid 900	Ethylene-diamine linoleic acid polyamide	Applied Science Laboratories, Ltd.
Chromosorb W, HMDS	Calcined diatomaceous earth, hexamethyldisilane treated	Johns-Manville
Fluoropak 80	Fluorocarbon support	Fluorocarbon Co.

The 10% SE-52 packing was prepared by dissolving 1 g. SE-52 in 40 cc toluene. Nine grams of solid support were then mixed in gently and the slurry spread out to dry for several hours. Neopentyl glycol succinate was dissolved in chloroform and the packing prepared as for SE-52. Acetone was employed to dissolve QF-1, while a 1:1 mixture of isopropanol and chloroform was used for Versamid 900. A combination of SE-52 and Versamid 900 column was prepared by first applying the Versamid 900. The SE-52 coating was then applied. This was possible because toluene does not dissolve Versamid 900. A commercially prepared column of 5% SE-30 on Chromosorb W, 60/80 mesh was also used with the "Hy-fi".

To facilitate uniform packing of coiled columns, they were subjected to vacuum, and were tapped gently as the packing material was being poured in. The columns were conditioned at least overnight at temperatures slightly above the operating temperature, with a slight amount of helium or nitrogen flowing. While in operation, the temperature of the oven was maintained at about 200° C and that of the injector block, 50 to 100° higher.

Indole Compounds

The indole compounds used in this study were obtained from K & K Laboratories, Inc. A check was made on the purity of the compounds by means of gas and paper chromatography. When gas chromatographed, all gave single peaks except indoleacetonitrile and indoleacetamide which gave additional peaks corresponding to indole and skatole. However, their appearance may be due to destruction of indoleacetonitrile and indoleacetamide in the column rather than the presence of contaminants. This was borne out by the fact that paper chromatography of these two compounds on a Whatman No. 1 paper, using isopropanol/ammonia/water (8:1:1) as a solvent produced single spots.

Esterification of Indole Acids

Two methods of esterification were tried. One involved the use of BF_3-methanol as described by Metcalfe and Schmitz (71) for fatty acids. Also, a lower concentration of BF_3, 60 gm/liter was tried for methylating indole acids. The esterification was

affected by simply adding BF_3-methanol to the acid and then injecting the mixture into the chromatograph. Because indole acids are more labile than fatty acids, no heat was applied to the reaction mixture. Propyl esters were obtained by substituting n-propanol for methanol.

In another method, diazomethane was the methylating reagent. It was prepared according to the method of DeBoer and Backer (24), except that about 5-10 gms of tolylsulfonylmethylnitrosamide was used to lessen the danger of explosion during its preparation. The ethereal diazomethane was added to the acids until a yellow color persisted. The excess diazomethane and ether was evaporated off in a fume hood and the residue was taken up with methanol.

Tryptamine and Tryptophan

Tryptamine was obtained commercially as a hydrogen chloride. It was liberated from hydrogen chloride by treating the salt with a slight excess of ammonia or sodium bicarbonate and extracting the tryptamine with ether. An acetyl derivative was prepared by refluxing tryptamine with acetic anhydride and pyridine as described by Brooks and Horning (17).

Tryptophan was esterified by treating it with 5% HCl in anhydrous methanol overnight at room temperature. The residue was taken up in methanol. Acetylating the ester with acetic anhydride was attempted in a manner similar to the procedure used for tryptamine.

Indole Aldehydes

Indolealdehyde and indoleacetaldehyde were chromatographed directly. Indoleacetaldehyde was obtained commercially as a sodium bisulfite salt. Sodium bisulfite was removed by addition of sodium carbonate in excess and the free aldehyde was extracted with ether (38). Preparation of acetal derivatives were attempted by the method similar to the one used by Gray (36) for fatty aldehydes. Because indole aldehydes are unstable, they were not refluxed. Indole-aldehyde was treated for 10 minutes to 3 hours, while indoleacet-aldehyde sodium bisulfite was treated overnight. After this period of treatment with HCl-methanol, the mixture was neutralized with ammonia or sodium carbonate. The excess undissolved sodium carbonate was removed by letting it settle out and the supernatant mixture was injected directly into the chromatograph. If ammonia was used to remove the excess HCl, a precipitate was formed and this was allowed to settle out.

Collection, Quantitation, Calculation and Reliability of Results

The effluent gas was collected with glass tubes, narrowed at one end to fit the outlet of the chromatograph. A plug of loose cotton soaked in methanol was placed in the glass tube. Also, collection was attempted with a teflon tube inserted into the outlet steel tube. The eluants were checked qualitatively by rinsing the collection tubes into cuvettes and obtaining the absorption spectra at 230 to 300 mμ with a Beckman DK1 spectrophotometer. A quantitative check was obtained by reading the absorbance at 280 mμ and comparing this with a calibrated curve.

The areas under the peaks on the gas chromatograph charts were determined by a triangulation method which involved multiplying the height by one-half of the base width. The N (theoretical plates) values were calculated from the formula $N = 16\ (t_x/Y)^2$ (43) where t_x = time for elution of peak and Y is the peak width. The peak width was taken as the segment of peak base intercepted by tangents to the inflection points on either side of the peak.

Reliability and reproducibility of results were determined by use of ethyl indoleacetate as a reference standard and by repeating the preparation procedures and separations. The more promising procedures were repeated four to ten times, whereas all others were tested at least twice.

For all quantitative determinations a uniform procedure of sample injection was employed. The full length of the needle was inserted and the sample was introduced slowly (10 seconds for a 10 µl sample). After the required amount of sample was injected, the syringe was quickly pulled out.

Preparation of Indole Extracts

Two methods of extraction of cabbage (Brassica oleracea var. capitata) were carried out. In one method, 200 g. of the outer leaves of a cabbage head were extracted with methanol and taken to dryness as described by Fletcher and Zalik (31). The residue was taken up in 2% sodium bicarbonate solution and partitioned with peroxide free diethyl ether. The ether layer, containing the neutral

indoles was taken to dryness and the residue was dissolved in 0.3 ml of methanol. Aliquots of 20 to 50 µl were injected into the gas chromatograph. This procedure was also tried on seedlings of beans (Phaseolus vulgaris, var. Dutch brown), broad beans (Vicia faba, var. Broad Windsor) and corn (Zea mays, var. Early Alberta).

In another procedure, ten day old seedlings of Savoy cabbage (13 g.) were harvested and freeze dried. The dried matter was pulverized and the indole compounds were extracted on a silica gel column (79). The eluates were evaporated in a water bath and the residue taken up in 0.3 ml of methanol. Aliquots of 20 µl were injected into the gas chromatograph.

RESULTS AND DISCUSSION

Comparison of Columns and Operating Conditions

The results obtained during the search for a suitable column and operating conditions will be discussed under the headings (a) column substrates, (b) temperature and (c) conditioning of the column.

(a) Column substrates

It was necessary, first of all to find a suitable packing for the column for the separation of indole auxins. The first attempts were made on the polar substrates, Versamid 900 and QF-1 coated on Chromosorb W and Chromosorb W, HMDS treated supports. Although Stowe and Schilke (92) reported good separation with these columns, we were less successful. Only a few indole derivatives gave a response with Versamid 900, while QF-1 resulted in much tailing of the eluant. This would suggest that Versamid 900 and QF-1 are reactive to the indole compounds.

A semi-polar substrate, SE-52 was, therefore, applied to Chromosorb W, HMDS and Fluoropak 80 solid supports. Fluoropak 80 is very inert but difficulty was experienced in filling the column with it because of plugging. Moreoever, high pressure of the carrier gas was necessary to obtain a reasonable flow rate with Fluoropak 80 and the efficiency was low. Ten per cent SE-52 on Chromosorb W, HMDS or Anakrom ABS proved to be the best combination for the widest range

of indole compounds. Good separations were also obtained with SE-30 on Chromosorb W or Anakrom ABS columns. Another substrate showing some promise for certain indole compounds was neopentyl glycol succinate. The relative retention times for the various indole derivatives on three different columns are presented in table II. Ethyl indoleacetate (92) was chosen as the standard of reference because it is stable over a wide temperature range, has an intermediate retention time, and is of interest to plant physiologists.

Table II. Relative Retention Times for Various Indole Derivatives on Three Different Columns

Retention times are relative to ethyl indoleacetate and are averages of 2-4 runs.

	A*	B*	C*
Indole	.16	.22	.11
Skatole	.21	.22	.11
Indoleacetaldehyde	.63	-	-
Tryptamine	.71	.69	-
Tryptophol	.78	.69	.88
Indoleacetonitrile	.86	.78	1.57
Indoleacetamide	.88	-	-
Indolealdehyde	.87	1.02	-
Ethyl indoleacetate	1.00	1.00	1.00

* Composition of columns and operating conditions used were:

A. 10% SE-52 on Chromosorb W, 60/80 mesh, HMDS; 6½' x ¼" stainless steel column; oven temperature, 195° C; injector block temperature, 280° C; thermal conductivity cell detector; carrier gas, helium at 45 cc per minute.

B. 5% SE-30 on Chromosorb W, 60/80 mesh; 5' x 1/8" stainless steel column; oven temperature, 200° C; injector block temperature, 270° C; hydrogen flame ionization detector; carrier gas, nitrogen at 10 cc per minute.

C. 5% Neopentyl glycol succinate on Chromosorb W, 60/80 mesh, HMDS; 2½' x ¼" stainless steel column; oven temperature, 205° C; injector block temperature, 280° C; thermal conductivity cell detector; carrier gas, helium at 90 cc per minute.

Based upon the retention times obtained for the indole derivatives when they were run individually on an SE-52 column, one would expect indoleacetonitrile, indoleacetamide and indolealdehyde to give one broad peak. An SE-30 on Chromosorb W column separates indoleacetonitrile from indolealdehyde satisfactorily but indoleacetamide and indoleacetaldehyde are destroyed with this column. With a neopentyl glycol succinate column, the retention time for indoleacetonitrile was higher than for ethyl indoleacetate while the reverse was true for the silicone columns. Stowe and Schilke (92) reported a relative retention time of 1.81 for indoleacetonitrile as compared to 1.57 obtained here. This is probably due to the difference in oven temperatures employed. Higher temperature would increase the gap between ethyl indoleacetate and indoleacetonitrile. A neopentyl glycol succinate column, however, destroyed the more labile indoles like tryptamine and the aldehydes.

Only the SE-52 column was able to separate indole from skatole. Identical retention times for indole and skatole were obtained for both the SE-30 and neopentyl glycol succinate columns.

To further assess the adequacy of these three columns, a mixture of indoles was chromatographed. The mixture consisted of tryptophol, indoleacetonitrile, indoleacetamide and ethyl indoleacetate. Five µl consisting of 50 µg of each indole was injected when the thermal conductivity cell was used as the detector, while 1 µl consisting of 1 µg of each indole was used with the hydrogen flame ionization detector. Typical chromatograms are shown in figures 1, 2 and 3.

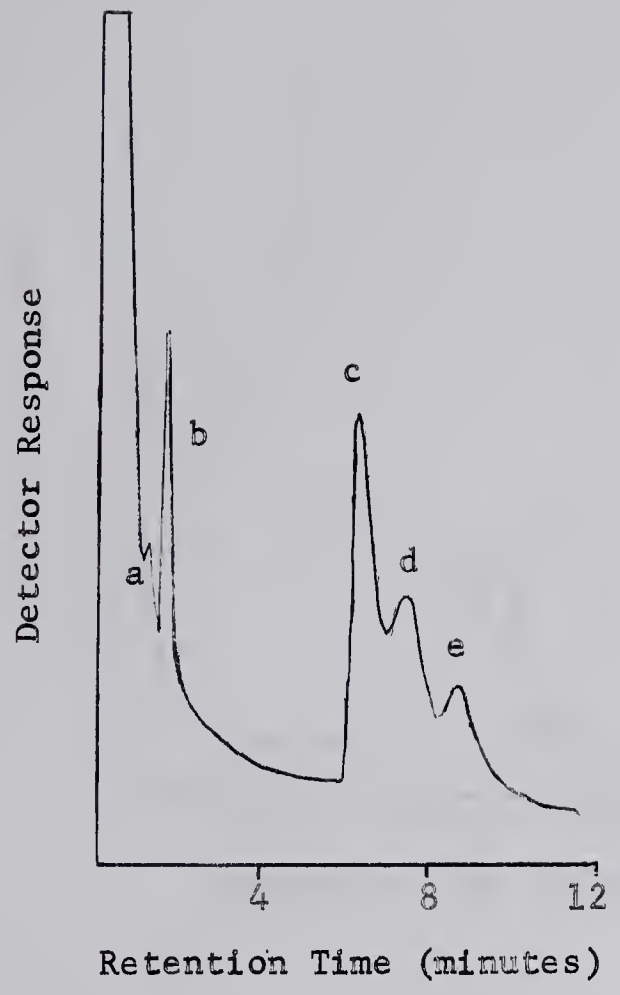


Fig. 1. Chromatogram of neutral indoles from a 6½' SE-52 column. ¼" Diameter stainless steel column, 10% SE-52 on Chromosorb W, HMDS. Oven temperature, 195° C. Thermal conductivity cell detector. Carrier gas, helium at 45 cc/min.

(a) indole
(b) skatole
(c) tryptophol
(d) indoleacetonitrile and indoleacetamide
(e) ethyl indoleacetate.

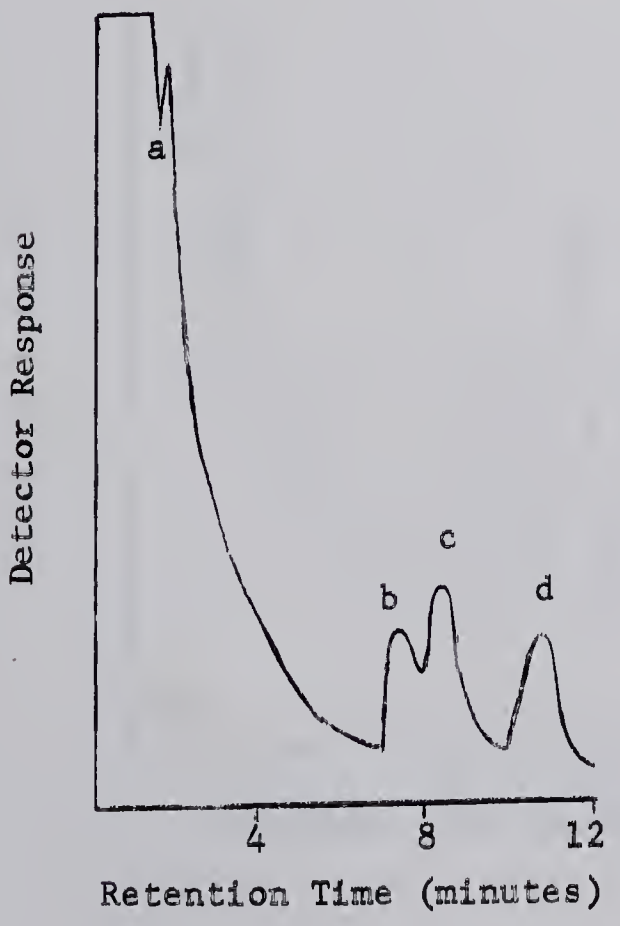


Fig. 2. Chromatogram of neutral indoles from a 5' SE-30 column. 1/8" Diameter stainless steel column, 5% SE-30 on Chromosorb W. Oven temperature, 200° C. Hydrogen flame ionization detector. Carrier gas, nitrogen at 10 cc/min.

(a) indole and skatole
(b) tryptophol
(c) indoleacetonitrile
(d) ethyl indoleacetate

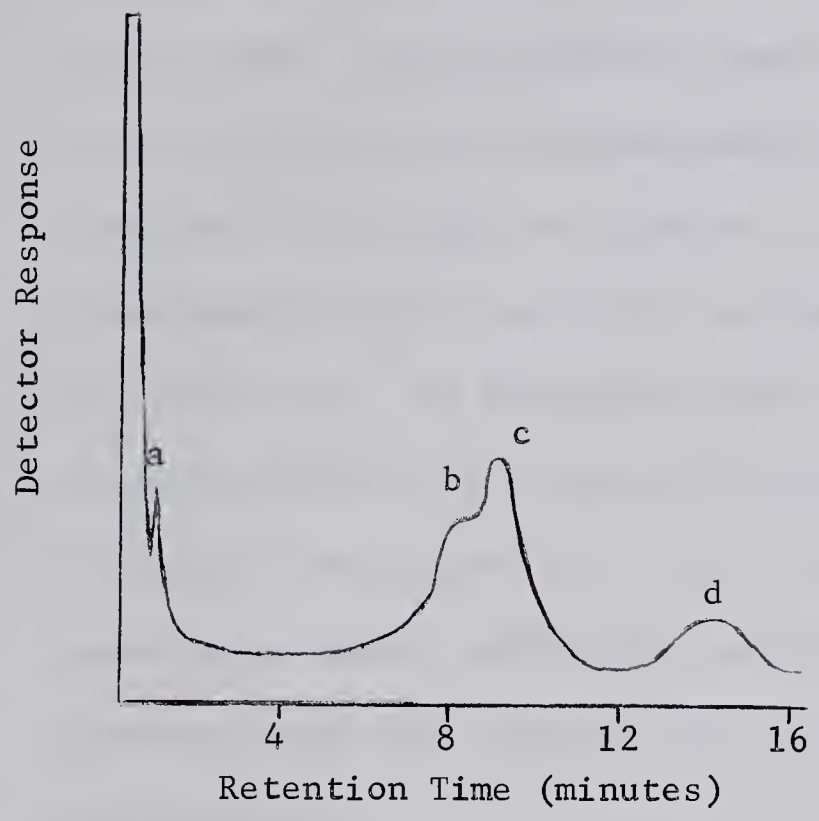


Fig. 3. Chromatogram of neutral indoles from a 2½' neopentyl glycol succinate column. ¼" Diameter stainless steel column, 5% neopentyl glycolsuccinate on Chromosorb W, HMDS. Oven temperature, 205° C. Thermal conductivity cell detector. Carrier gas, helium at 90 cc/min.

(a) indole and skatole
(b) tryptophol
(c) ethyl indoleacetate
(d) indoleacetonitrile

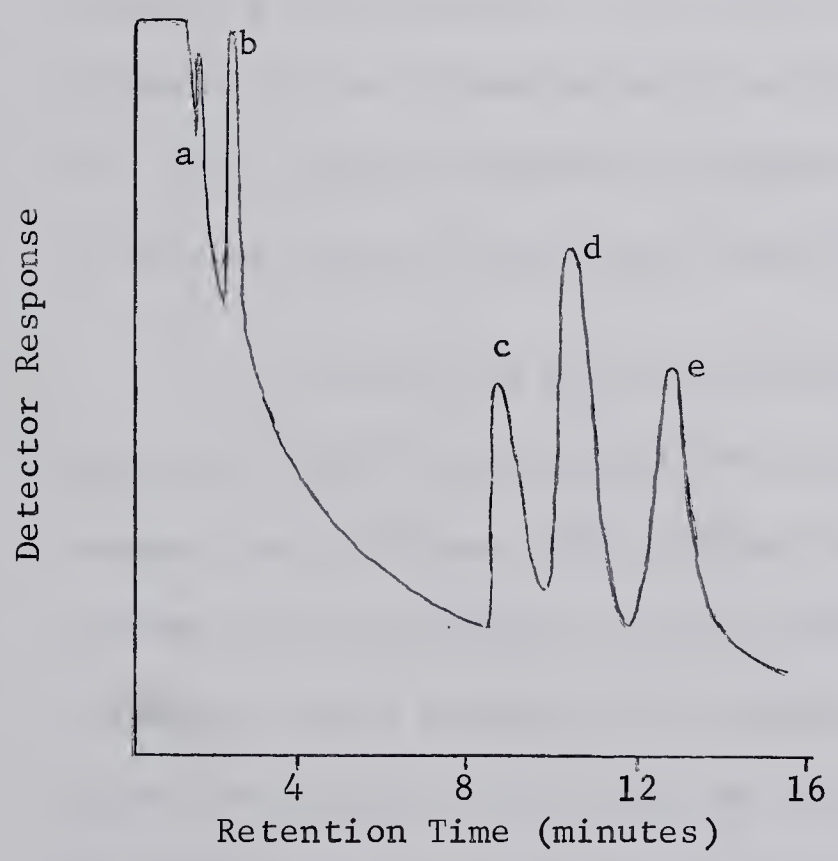


Fig. 4. Chromatogram of neutral indoles from a 9' SE-52 column. 1/8" Diameter stainless steel column, 10% SE-52 on Anakrom ABS. Oven temperature, 205° C. Hydrogen flame ionization detector. Carrier gas, nitrogen at 10 cc/min.

(a) indole
(b) skatole
(c) tryptophol
(d) indoleacetonitrile
(e) ethyl, indoleacetate

It can be seen that all indoles gave a response with the SE-52 column. However, indoleacetamide and indoleacetonitrile appeared as one peak. Also, complete separation of the four indoles, tryptophol, indoleacetonitrile, indoleacetamide and ethyl indoleacetate was not obtained. The SE-30 on Chromosorb W gave a better separation of indoleacetonitrile and ethyl indoleacetate, but indoleacetamide was not detected. The neopentyl glycol succinate column gave an excellent separation of ethyl indoleacetate and indoleacetonitrile, while tryptophol overlapped with ethyl indoleacetate. Separate peaks corresponding to indole and skatole were obtained only with the SE-52 column, whereas SE-30 and neopentyl glycol columns yielded single peaks as was expected.

Since a longer column increases the efficiency (theoretical plates) proportionally, a nine foot SE-52 column was tried. Figure 4 shows a typical chromatogram from this column. It can be seen that the three indole compounds, tryptophol, indoleacetonitrile and ethyl indoleacetate were separated completely.

As has been mentioned under Materials and Methods, a combination column of SE-52 and Versamid 900 can be obtained by first coating the support with Versamid 900, followed with a coating of SE-52. This column was tried because it was thought that Versamid 900 being a polar substrate would increase the retention time of the more polar indoles. Also, the Versamid 900 covers up the active sites of the support and for this reason it has proven to be satisfactory for separation of steroids (53). The relative retention time and N values (effective

plate values) of neutral indoles and methyl esters of indole acids from this packing are compared with those for SE-52 in table III.

Table III. Comparison of Relative Retention Times (r) and Effective Plate Values (N) for an SE-52 Column and a Combination of SE-52 and Versamid 900

Retention times are relative to ethyl indoleacetate. Thermal conductivity cell was used as the detector in both cases. Results are averages of 2-4 runs.

	A*		B*	
	r	N	r	N
Indole	.16	350	.19	400
Skatole	.21	360	.25	300
Indoleacetaldehyde	.63	520	.67	340
Tryptophol	.78	150	.94	40
Indoleacetonitrile	.86	320	1.10	150
Methyl indoleacetate	.83	560	.83	190
Methyl indolepropionate	1.12	560	1.06	185
Methyl indolebutyrate	1.59	560	1.49	170
Ethyl indoleacetate	1.00	625	1.00	210

* Composition of columns and operating conditions used were:
A. 10% SE-52 on Chromosorb W, HMDS, 60/80 mesh, 6½ feet. Oven temperature, 195° C. Injector temperature, 280° C. Helium flow rate at 40 cc/min.
B. 0.2% Versamid 900 and 5% SE-52 on Chromosorb W, HMDS, 60/80 mesh, 6 feet. Oven temperature, 197° C. Injector temperature, 290° C. Helium flow rate at 50 cc/min.

A slight coating of Versamid 900 underneath SE-52 made the column more polar and thus increased the retention time of the more polar compounds. This was particularly noticeable with tryptophol

and indoleacetonitrile whose relative retention times have been increased from .78 to .94 and from .86 to 1.10 respectively. Versamid 900 however decreased the efficiency of the column. It can be seen that the effective plate values have been considerably decreased for all the indole compounds except indole and skatole and with a 6 foot column, most of the indole compounds came out as one broad peak. Higher oven temperatures or a longer column might have improved the efficiency of this column. However, either of these conditions may also destroy some of the indole compounds, and even under the conditions that were employed, the doubly coated column did not elute indoleacetamide and tryptamine.

The column substrates and conditions used for separation of indole auxins have been summarized in table IV. The silicone columns have been found to be the most versatile. Both SE-52 and SE-30 when coated on a silanized diatomaceous earth support proved to be satisfactory for most indoles. Fluoropak 80 as a solid support required a high pressure to obtain a reasonable carrier gas flow rate and as a result only the compounds with short retention times like indole and skatole could be detected. A slight undercoating of Versamid 900, while it increased the retention time of some indoles, decreased the efficiency of the column. QF-1 like the other silicones gave peaks for most of the indoles, but because of its greater polarity, much tailing resulted and some of the polar indoles were destroyed. Neopentyl glycol succinate was found suitable for separation of indole-acetonitrile from most other indoles. Its drawback was that the retention time was high and some of the indoles were destroyed.

Table IV. Column Substrates and Conditions Used for Separation of Indole Auxins

Liquid Phase	Solid Support	Length	Oven Temperature	Flow Rate (cc/min.)	Compounds Detected	Remarks
* 5% SE-30	Chromosorb W	5'	205° C	10	Most indoles	Satisfactory
10% SE-30	Anakrom ABS	6'	210° C	40	Most indoles	Satisfactory
10% SE-52	Chromosorb W, HMDS	6½'	205° C	40	Most indoles	Satisfactory
*10% SE-52	Fluoropak 80	9'	205° C	10	Most indoles	Satisfactory
** 3% SE-52	Chromosorb W, HMDS	6'	209° C	40	Indole, skatole	Poor
0.2% Versamid 900 and 5% SE-52	Chromosorb W, HMDS	6'	197° C	50	Indole esters, IAN, tryptophol	Fair
**10% QF-1	Chromosorb W, HMDS	4'	210° C	60	Most indoles	Fair
10% Versamid 900	Chromosorb W, HMDS	5'	220° C	115	Indole, indole esters	Poor
5% Neopentyl glycol succinate	Chromosorb W, HMDS	2½'	205° C	90	Indole esters, IAN, tryptophol	Fair
Craig succinate	Firebrick	10'	235° C	90	Indole, indole esters	Fair

* Hydrogen flame ionization detector was used. Thermal conductivity cell detector was used for all other columns.

** Copper columns were used. All others were stainless steel.

Attempts to separate indole compounds with a Versamid 900 or Craig succinate column were not successful.

At first, copper tubing was used for the columns, but it was thought that this might catalyze the destruction of indole compounds. Therefore a switch was made to stainless steel columns.

Most of the indole compounds were detected with a thermal conductivity cell. However, at least 10 µg of indole compound had to be injected for detection with it. The more promising columns were then tried with a hydrogen flame ionization detector which was found to be at least 100 times more sensitive than the thermal conductivity cell.

(b) Temperature

In order to establish the most suitable temperature for the SE-52 column, runs of four different indole compounds were made at three different temperatures. The effective plate values were then calculated and these are presented in table V.

Table V. The Effect of the Oven Temperature on Efficiency (Theoretical Plates) of an SE-52 Packing

The column used was 10% SE-52 on Anakrom ABS, 9' x 1/8" stainless steel. Hydrogen flame ionization detector was used with nitrogen as a carrier gas at 10 cc per minute.

	Indole Compound			
Temperature	Ethyl indoleacetate	Indole-acetonitrile	Indolealdehyde	Tryptophol
190° C	640	158	76	74
205° C	1220	166	320	130
220° C	1060	160	140	95

It can be seen that the temperature had an effect on the efficiency of the column for all indole compounds except indoleacetonitrile. The maximum efficiency was obtained with an oven temperature of 205° C when an SE-52 packing was used. Besides oven temperature, the temperature of the injector block was found to be an important factor. For efficient separation of indoles, it was found necessary to have the injector block at least at 250° C, 50-100° above that of the oven.

(c) Conditioning of column

Figure 5 illustrates the effect of conditioning of the column on its ability to separate indole compounds. The chromatograms are typical for the more labile compounds like tryptophol and indoleacetamide. Before an SE-52 column could be used routinely to give reproducible results, it was necessary to condition it for a few days and run a series of indole samples through it. The conditioning is believed to remove the active sites on the column and reduces bleeding. Similar observations were reported by other workers. Brooks (16) in gas chromatography of corticosteroids and Farquhar (26) with fatty aldehydes reported response of certain compounds only after the columns were aged for a few days.

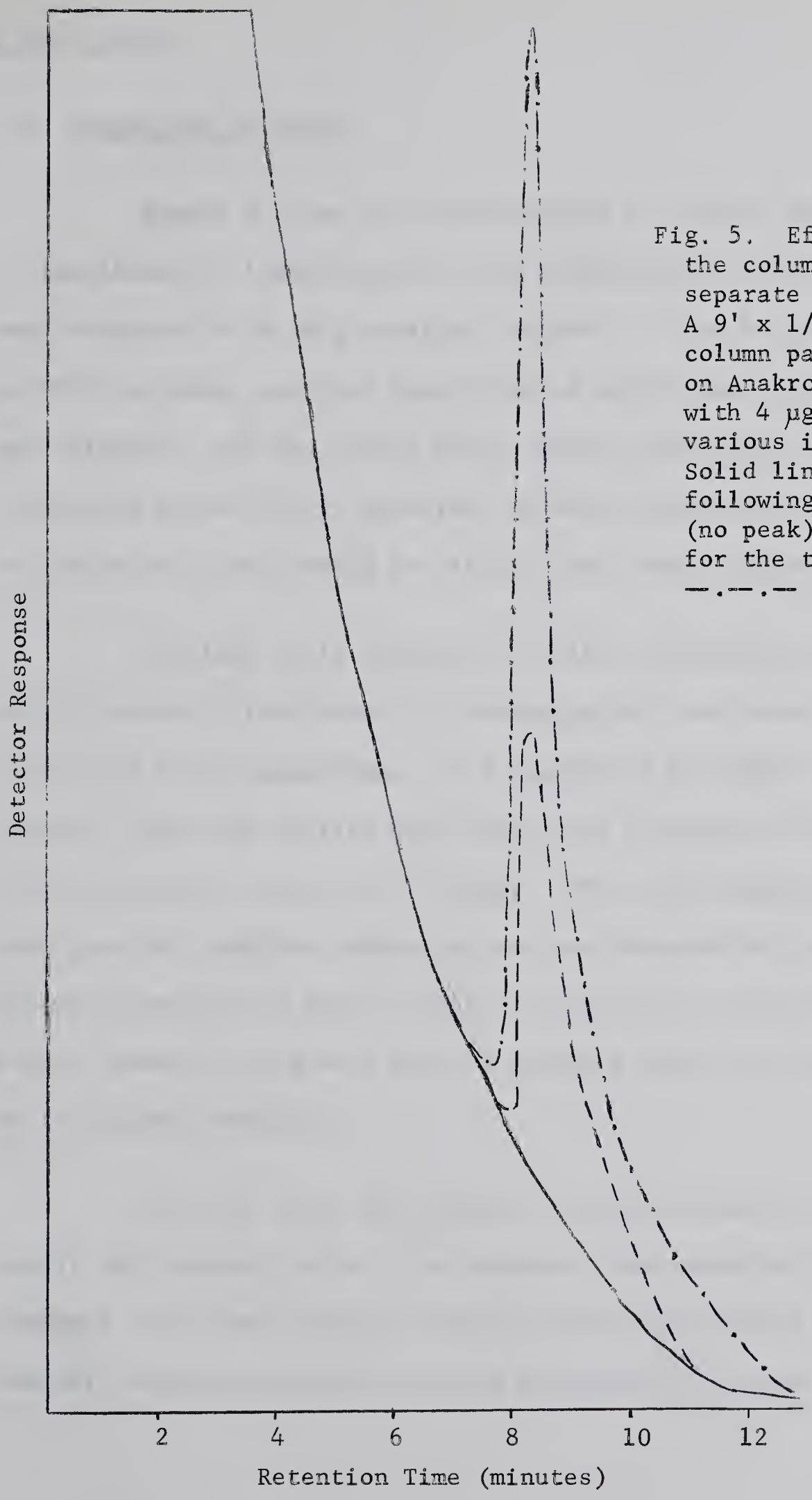


Fig. 5. Effect of conditioning the column on its ability to separate indole compounds. A 9' x 1/8" stainless steel column packed with 10% SE-52 on Anakrom ABS was injected with 4 μg of tryptophol at various intervals after packing. Solid line, first injection following overnight conditioning (no peak). ---- chromatogram for the tenth injection. —.—.— four days later.

Indole Acids

(a) Comparison of esters

Figure 6 shows the chromatograms for methyl and n-propyl esters of indoleacetic, indolepropionic and indolebutyric acids. The esters were obtained by the BF_3 catalysis method. It can be seen that with an SE-52 packing, complete separation of methyl and n-propyl esters was obtained. All the indole acids except indolelactic could be esterified either by BF_3 catalysis or with diazomethane. Esterification of indolelactic acid could be effected only with diazomethane.

Williams (111) obtained a similar chromatogram showing the methyl esters of indoleacetic, indolepropionic and other urinary acids esterified with diazomethane. The separation was done on a 2% SE-30 column. Stowe and Schilke (92) have tried a series of esters; methyl, ethyl, n-propyl, n-butyl and 2-butyl. They were prepared by BF_3 catalysis and complete separation was not obtained with a Versamid 900 column of methyl and ethyl esters of indoleacetic and indolepropionic acids. However, they were able to separate them as n-propyl esters or its higher homologues.

Table VI lists the relative retention times of several methyl and n-propyl esters. As expected, the retention times of the n-propyl esters were greater than for the corresponding methyl esters. However, excellent separations were achieved with either ester.

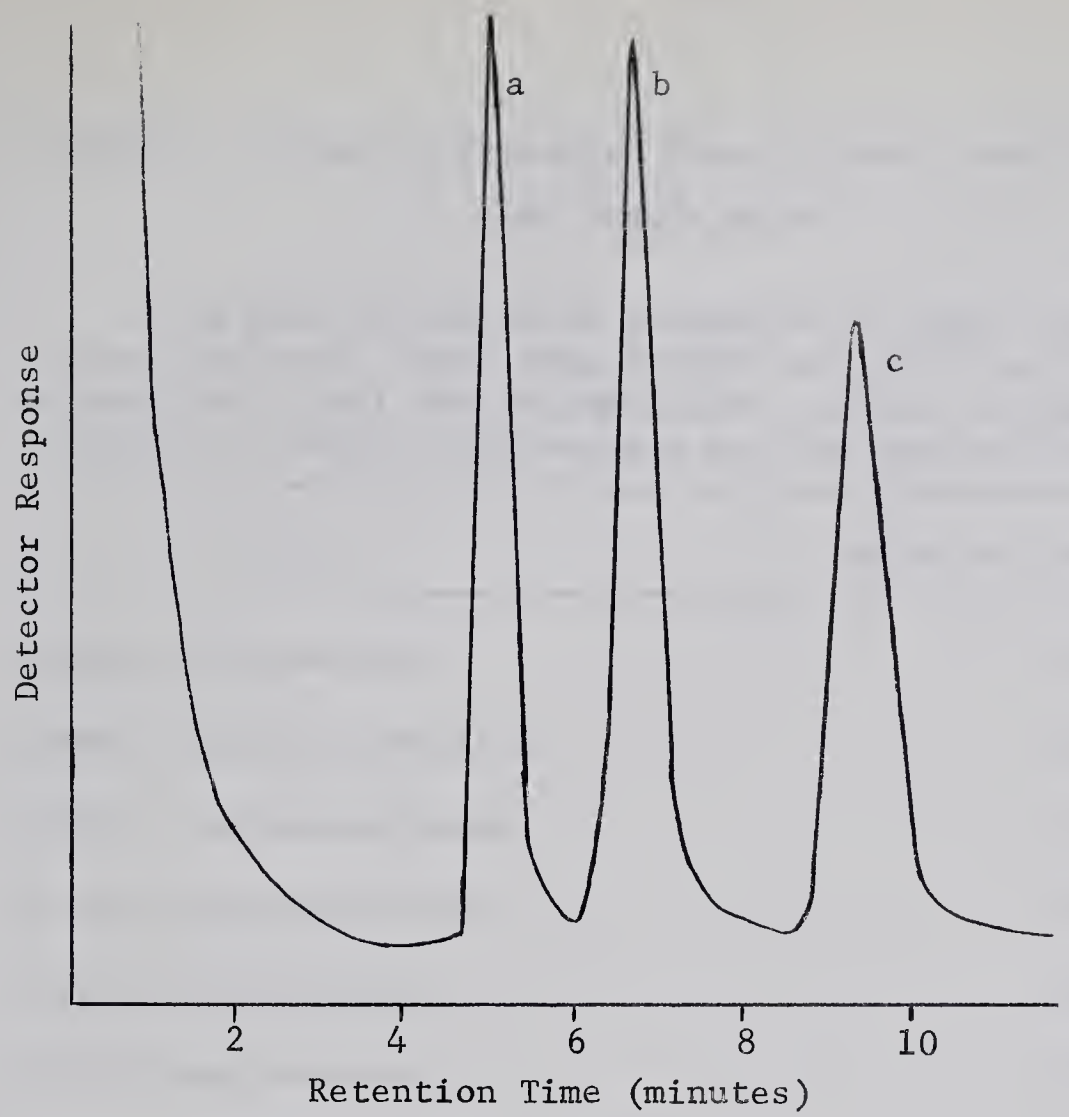


Fig. 6. Chromatograms of indole acid esters. A 6½' x ¼", 10% SE-52 Chromosorb W, HMDS, stainless steel column was used with oven temperature at 200° C and thermal conductivity cell as the detector. Top, methyl esters. Bottom, n-propyl esters. (a) indoleacetate, (b) indolepropionate, (c) indolebutyrate.

Table VI. Relative Retention Time of Methyl and Propyl Esters of Indole Acids

A 6½ foot 10% SE-52 on Chromosorb W, HMDS, stainless steel column was used. Oven temperature was 195° C and thermal conductivity cell was the detector. Retention times are relative to ethyl indoleacetate and are averages of 3-4 runs.

Ester	Relative Retention Time
Methyl indoleacetate	0.83
Methyl indolecarboxylate	0.96
Methyl indolepropionate	1.12
Methyl indolebutyrate	1.59
Methyl indolelactate	1.93
Ethyl indoleacetate	1.00
n-Propyl indoleacetate	1.40
n-Propyl indolepropionate	1.95
n-Propyl indolebutyrate	2.85

(b) Methods of esterification

The two methods of esterification were compared for their efficiency. Table VII shows the peak areas obtained by the two methods of esterification of 200 µg of indoleacetic acid at different times after treatment with the esterifying reagent.

Table VII. Comparison of Efficiency of Two Methylation Procedures of Indoleacetic Acid

Relative peak areas represent the ester of 200 μg of indoleacetic acid at different times after esterification and are averages of 2-4 runs. A 10-foot 10% SE-52 on Anakrom ABS, stainless steel column was used. Oven temperature was 205° C and a thermal conductivity cell was the detector.

Time after Esterification	Method of Esterification	
	Diazomethane	BF_3-methanol
0		15.2*
10 minutes	23.1	23.9
90 minutes		21.9
15 hours	24.3	21.9
36 hours	22.5	21.3

* Significantly different at 1% level

Esterification of indole acids with BF_3 as a catalyst had the disadvantage that it produced colored solutions. This has also been reported by other workers (92, 111) who stated that it resulted in some loss of the indole acid. At first, the BF_3 concentration used was 125 grams per liter as reported by Metcalfe and Schmildt (71) in the esterification of fatty acids. But since this concentration of BF_3 produced a high degree of color and resulted in partial loss of the ester, a weaker concentration of BF_3 (60 g./liter) was used for the comparison.

It is clear that there is no significant difference between the two methods of esterification. In the case of BF_3-methanol esterification, only 10 minutes of treatment was enough. Although it was thought that excess BF_3 might destroy the methyl ester upon standing, no significant loss had occurred after 36 hours. Ester formed by diazomethylation was found to be stable for several months.

Indole Aldehydes

The indole aldehydes, which include indolealdehyde and indole-acetaldehyde were successfully chromatographed on a SE-52 column directly. Acetal derivatives which are more stable to heat were obtained by Gray (36) for fatty aldehydes. An attempt was made to produce acetal derivatives of indole aldehydes by refluxing with 1% HCl in anhydrous methanol. Indolealdehyde particularly formed an intense color with this treatment. Therefore, the HCl-methanol treatment was done under mild conditions; room temperature for short time periods. After 20-60 minutes, the HCl was neutralized either with 1 N ammonia or sodium carbonate. The excess was indicated by the disappearance of the red color. Indoleacetaldehyde was treated with HCl-methanol as a bisulfite and then neutralized with excess of ammonia or sodium carbonate.

At first, the excess HCl was neutralized with ammonia, but it was found later that acetal hydrolysis can easily occur in an aqueous solution. Figure 7 shows the chromatograms after employing the two methods of neutralization in the acetalation of indolealdehyde. When the HCl-methanol and indolealdehyde mixture was neutralized with

ammonia, only one peak appeared (Fig. 7a), but when the mixture was neutralized with sodium carbonate two peaks occurred (Fig. 7b). The eluate corresponding to the peaks in figure 7 were collected and their absorption spectra were read to compare the eluants with authentic indolealdehyde. The eluate from the sample which was neutralized with ammonia had an absorption spectrum identical with indolealdehyde (Fig. 8a). No acetal derivative was formed. Based upon the absorption spectrum (Fig. 8b) the eluant of the first peak from the sodium carbonate neutralized sample was regarded as some form of derivative, probably an acetal. The second peak corresponded to unreacted indolealdehyde. The derivative was more volatile than the aldehyde itself, since its retention time was less.

A derivative of indoleacetaldehyde was prepared in a similar manner. Only one peak appeared when the mixture was neutralized with ammonia or sodium carbonate. This component was collected and its absorption spectrum was compared with that of indoleacetaldehyde (Fig. 9). It can be seen that the indoleacetaldehyde was transformed into a derivative. As in the case of indolealdehyde, the nature of the derivative was not investigated.

Further work would be necessary to improve procedures in preparing an acetal derivative of indole aldehydes before this method could be used to identify them routinely. Formation of indole aldehyde derivatives does not appear to be quantitative hence direct chromatography of indole aldehydes may be preferred. However, indole aldehyde derivatives may have some advantage in that their retention times

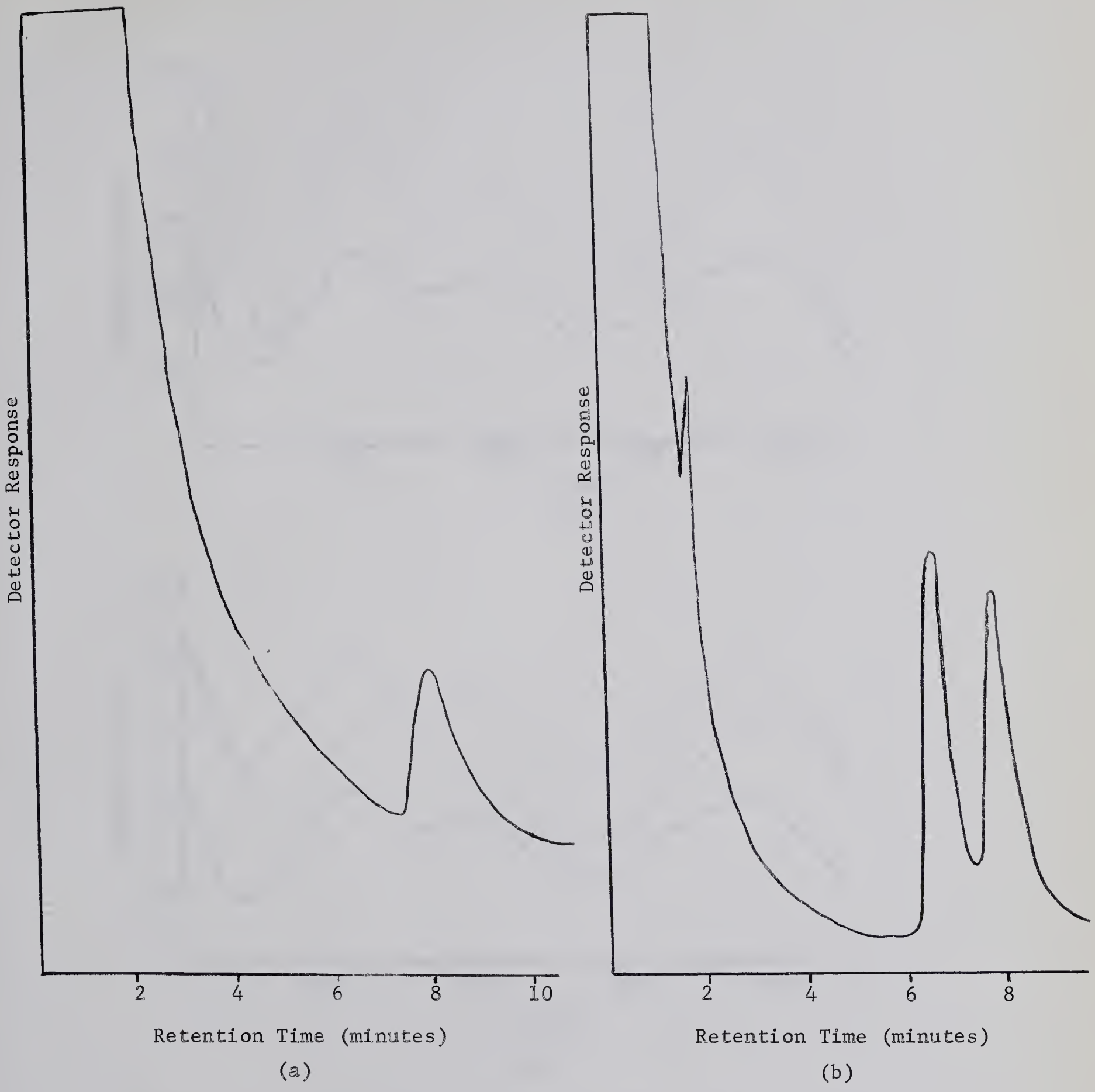
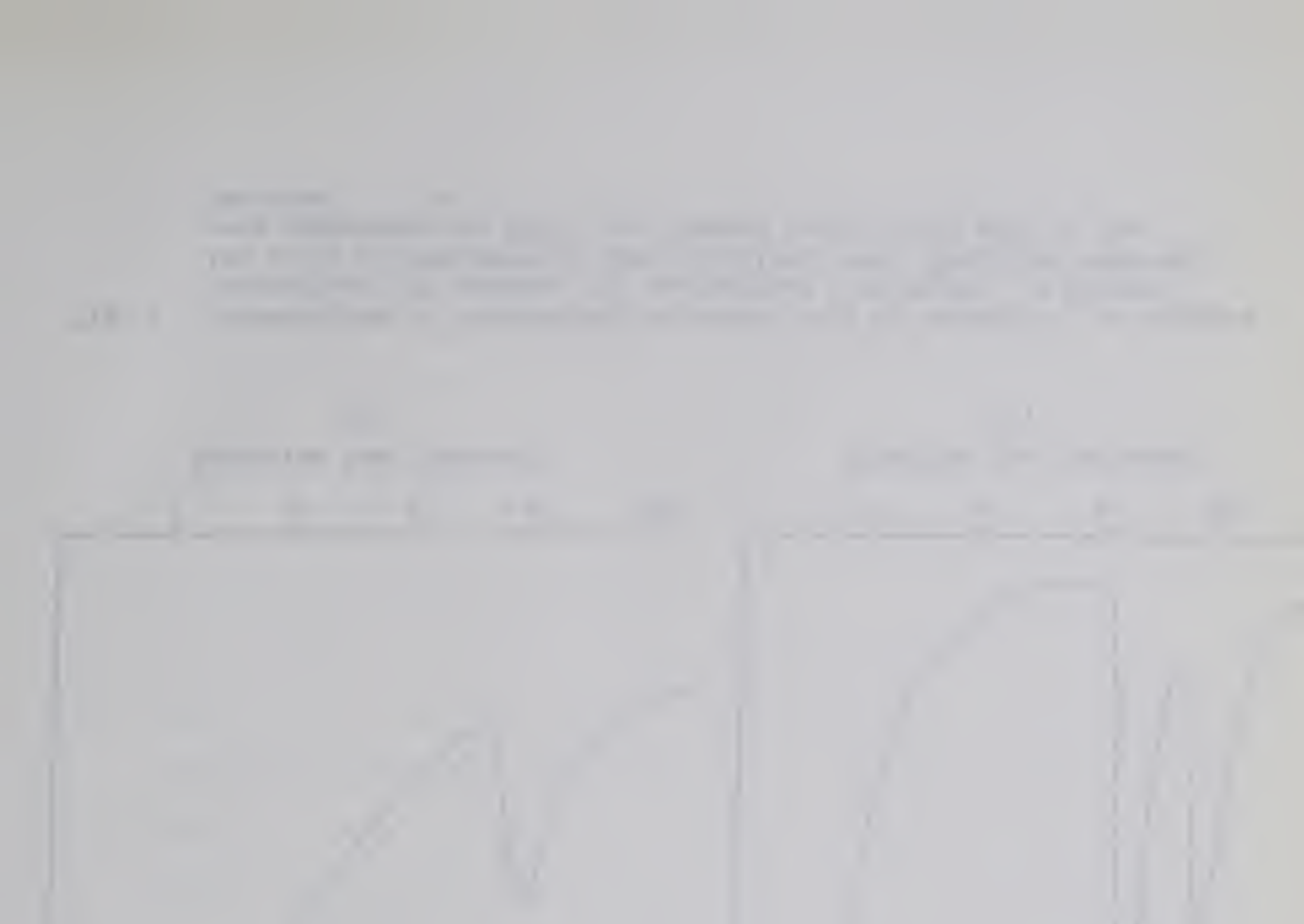
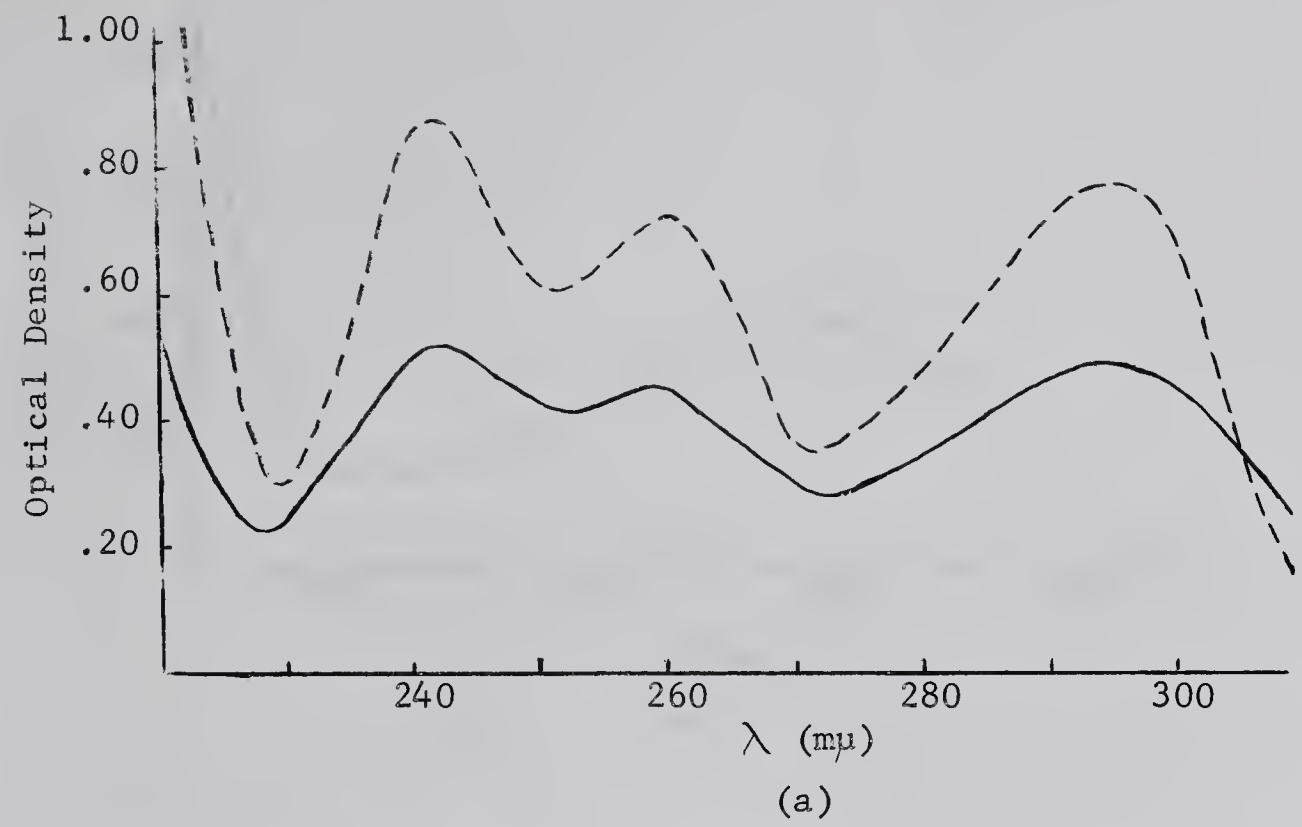
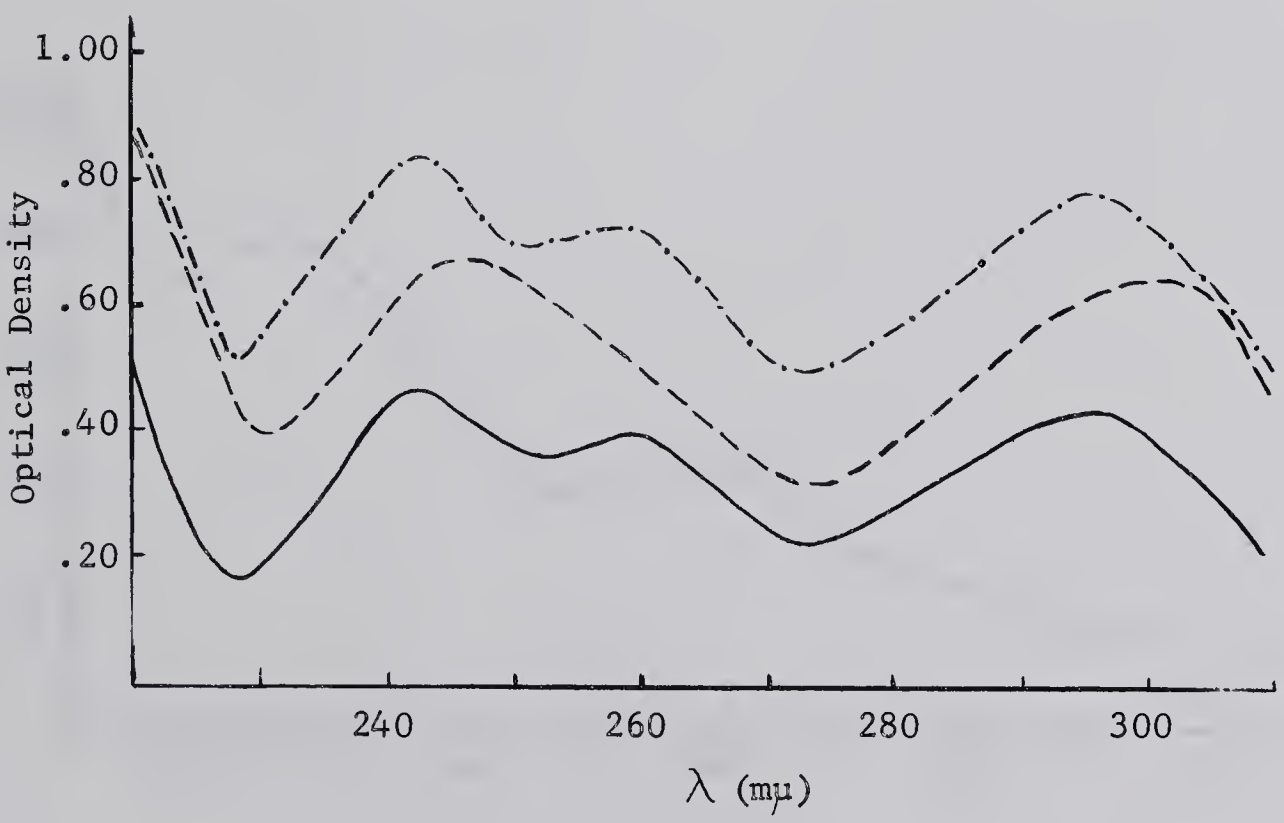


Fig. 7. Chromatograms of indolealdehyde treated with HCl-methanol. (a) mixture neutralized with ammonia, (b) neutralized with Na_2CO_3. A 6½-foot, 10% SE-52 on Chromosorb W, HMDS stainless steel column was used with oven temperature at 205° C and thermal conductivity cell as the detector.



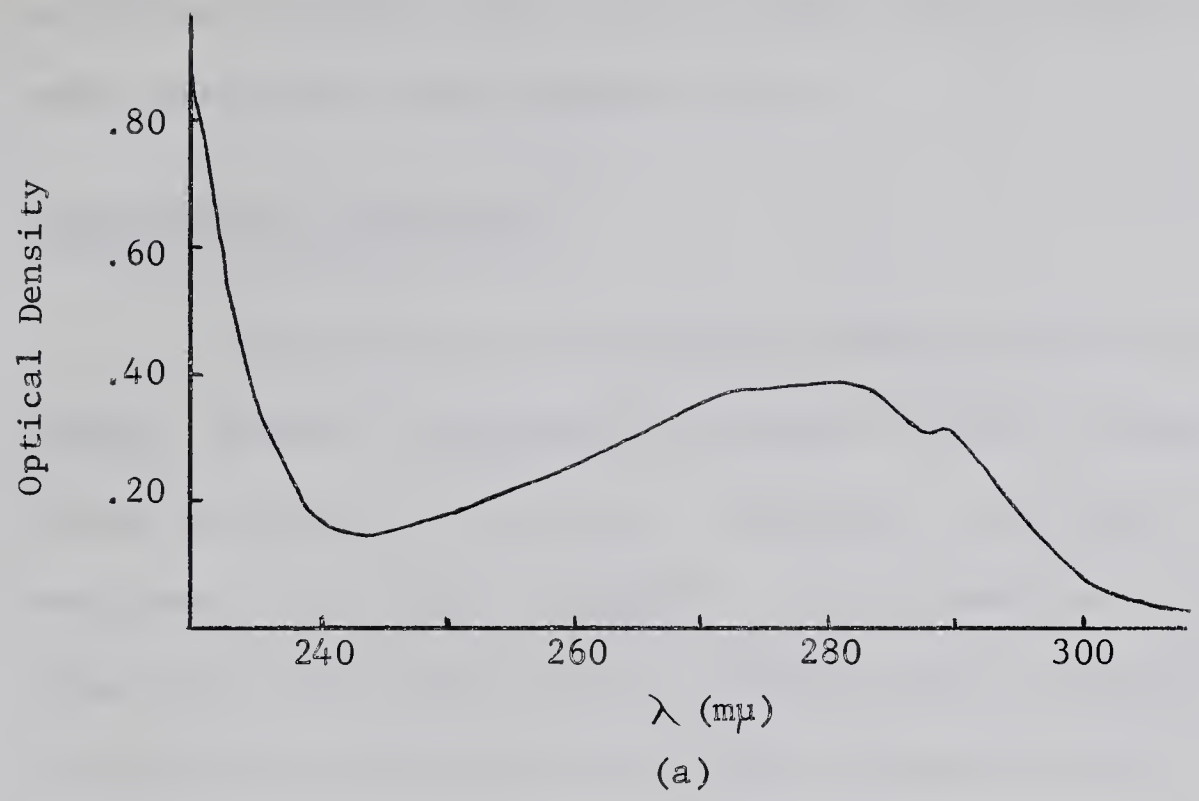


(a)

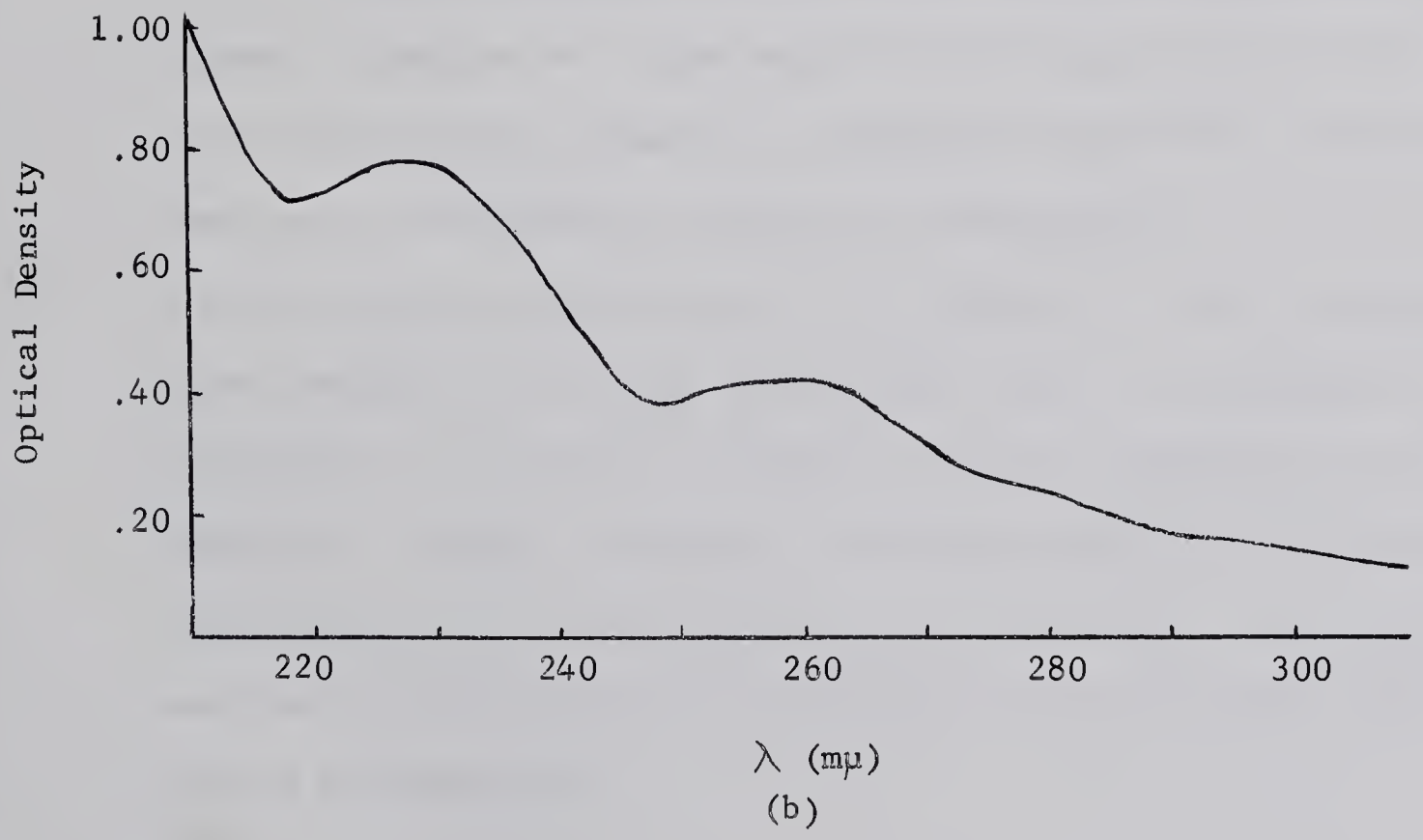


(b)

Fig. 8. Absorption spectra for indolealdehyde and its derivative collected as effluents from the chromatograms shown in fig. 7. (a) ——— authentic indolealdehyde, ------- effluent of peak in fig. 7a. (b) _____ authentic indolealdehyde, ------- first peak in fig. 7b, — — , —·—· second peak.



(a)



(b)

Fig. 9. Absorption spectra of indoleacetaldehyde and its derivative.
(a) indoleacetaldehyde
(b) derivative of indoleacetaldehyde when treated with HCl-methanol.

are changed and this may shift them away from the region where the peaks for other indole compounds appear.

Tryptamine and Tryptophan

Tryptamine was successfully chromatographed on an SE-52 column. Because tryptamine has a polar functional group, there is strong tendency for it to tail. Therefore, an attempt was made to acetylate it with acetic anhydride. This protected the labile amino group but at the same time, the retention time was greatly increased. The retention time relative to ethyl indoleacetate was .71 for tryptamine and 3.00 for its acetyl on an SE-52 column.

Since tryptophan itself is not volatile enough to be chromatographed, formation of a more volatile derivative was necessary. A methyl ester of tryptophan prepared by treating it with 5% HCl overnight, was found to be sufficiently volatile to chromatograph on an SE-52 column but its retention time was long; 5.46 relative to ethyl indoleacetate. Brooks prepared an N-acetyl amino methyl ester of tryptophan by first esterifying with 5% HCl in methanol and then acetylating with acetic anhydride. Attempts to prepare and chromatograph the N-acetyl amino methyl ester on the SE-52 column was not successful. This may have been due to destruction of the derivative, or more likely failure to produce the derivative.

Estimation and Recovery

For quantitation purposes, 25, 50, 70 and 100 µg of ethyl indoleacetate were injected. Four injections were made of each amount

and the average peak area was calculated. The quantity of ethyl indoleacetate was plotted against peak area as shown in figure 10. A linear relationship was found between the amount of ethyl indoleacetate injected and the peak area. This indicates that loss due to adsorption to the column was small, otherwise the relative loss would have been greatest for the smallest quantity.

Also the gas eluants were collected and determined quantitatively by ultra-violet absorption with a Beckman DK-1 spectrophotometer. A calibration curve was obtained for ethyl indoleacetate by reading the absorbance of the same amount that was injected into the chromatograph. The amounts collected varied from 64 to 71% when 50 to 100 μg quantities were used. The recoveries were similar to those obtained by other workers. The poor recoveries have been generally attributed to the formation of aerosol particles (50, 100) which cannot be condensed in an ordinary glass tube or Teflon tubing.

Other losses besides formation of aerosol particles may occur. This study was done on the Aerograph Model 90-S, and the loss could be due to design of the injector block or the collector block. Also, it was found that the area of the peak depended on how the sample was injected. The best response was obtained when the injection was made slowly. A fast injection probably caused a high buildup of pressure in the injector block and some of the samples may have been forced into the carrier gas line.

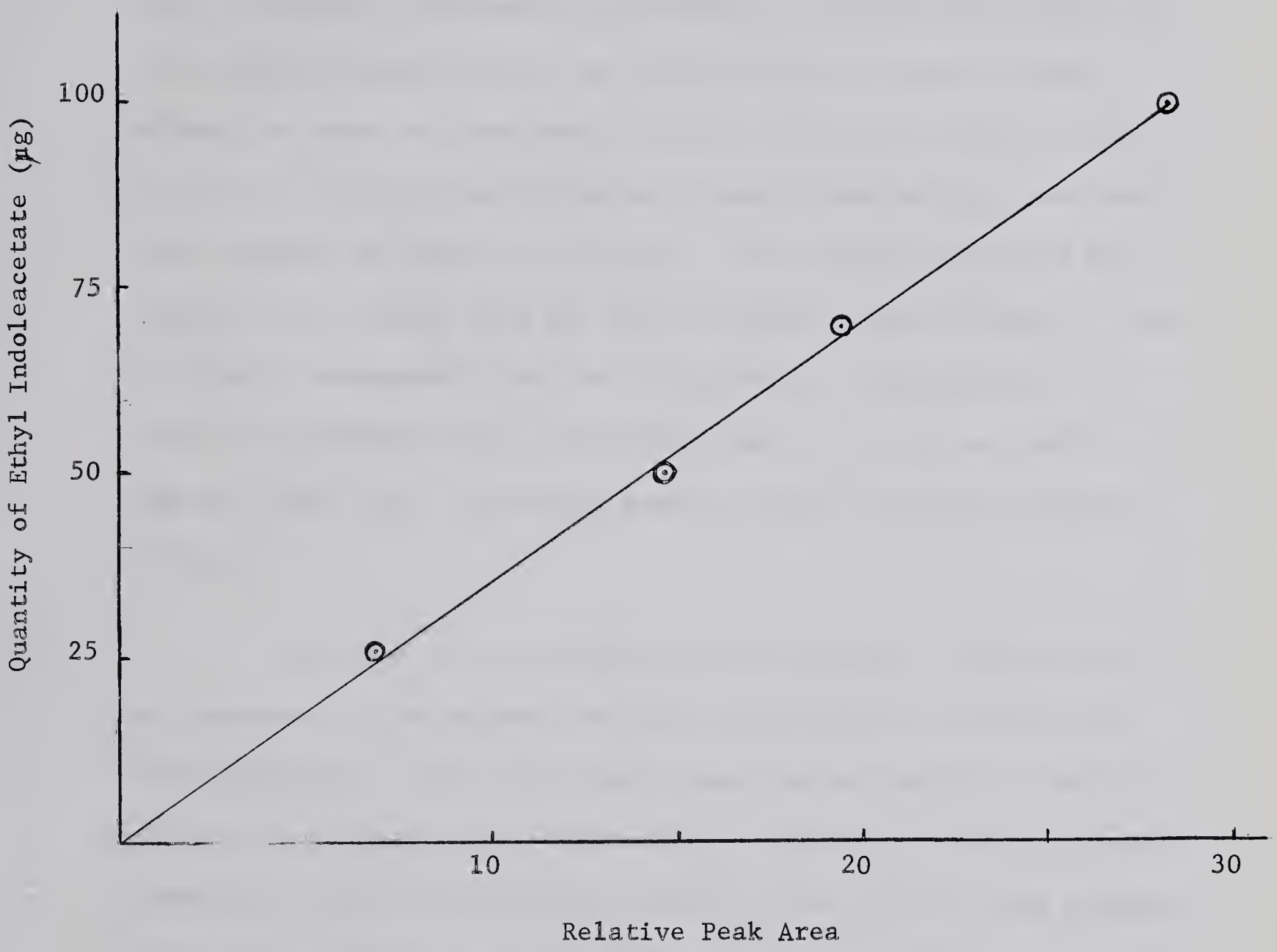


Fig. 10. Relationship between the quantity of ethyl indoleacetate injected and the peak area. A 6½-foot, 10% SE-52 on Chromosorb W, HMDS stainless steel column was used, with oven temperature of 205° C and thermal conductivity cell as the detector. The peak areas are averages of 4 runs.

Application of Gas Chromatography of Indole Auxins

An attempt was made to apply the technique of gas chromatography in isolating natural auxins from plants. Cabbage has been known to contain relatively large amounts of indoleacetonitrile and other growth hormones (64). An extract from seedlings of Savoy cabbage was made and fractionated with a silica gel column. When the neutral fraction was subjected to gas chromatography, two sharp peaks appeared as shown in figure 11. The retention times of both compounds were higher than any of the neutral indoles studied. When the eluate corresponding to the first peak was collected and the absorption spectrum read, it was identical to that of an indole compound (Fig. 12). The second peak was found to be a non-indolic substance.

Methanol extracts prepared from individual cabbage heads; one purchased in the market, the other fresh from the garden were chromatographed. These gave single peaks corresponding to the first peak in the chromatograms of the cabbage seedlings. The absorption spectrum for this substance was similar to that of an indole compound (Fig. 12). The discrepancy could have been due to the presence of a contaminant.

The indole compound isolated from the cabbage heads and seedlings may be one of the unidentified growth hormones mentioned by Linser (64) or it may be ascorbigen or "substance C" as reported by Kutáček in Savoy cabbage.

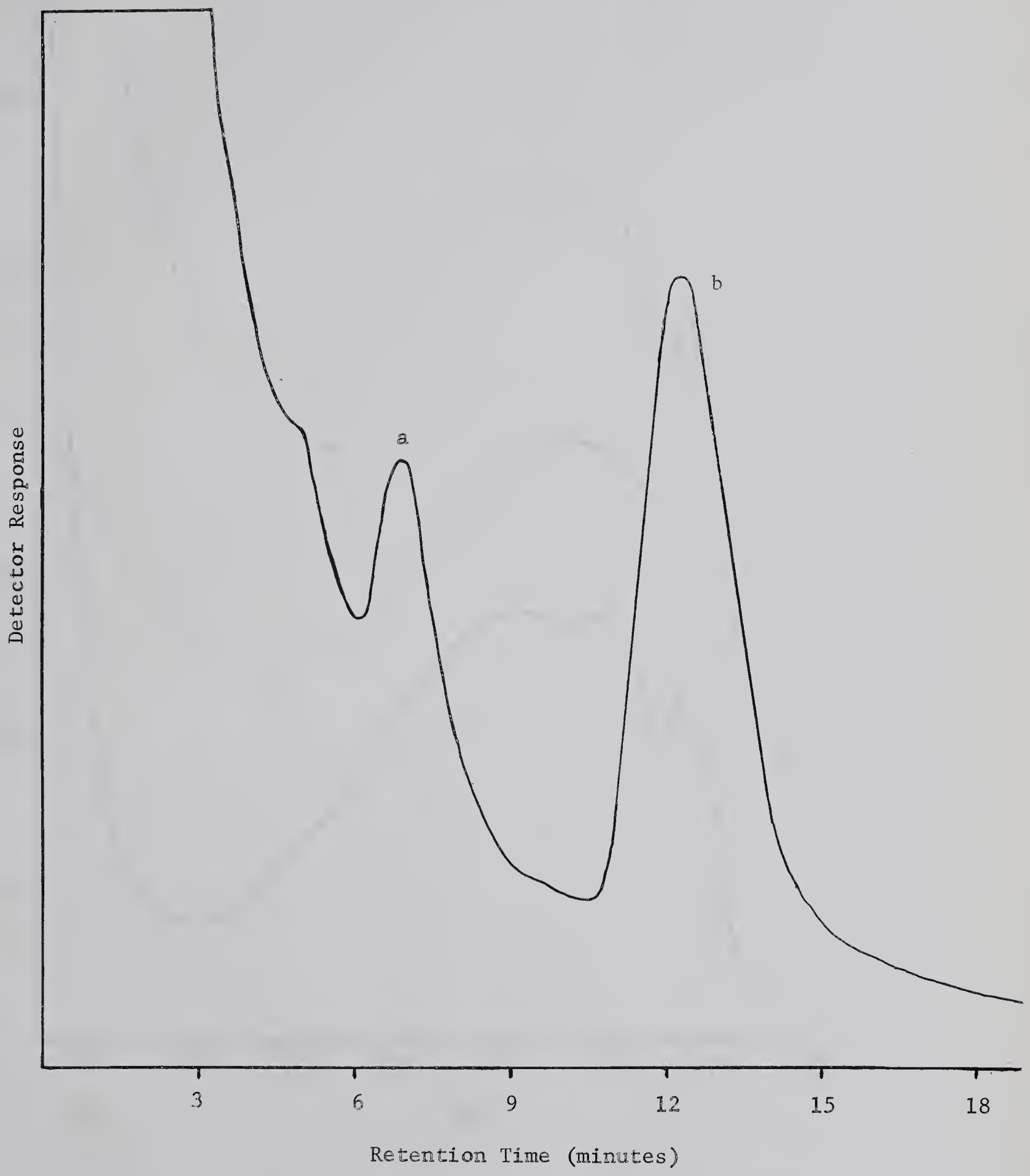


Fig. 11. Chromatogram of cabbage seedlings extract. A 6-foot, 10% SE-30 on Anakrom ABS, stainless steel column was used, with oven temperature of 205° C and thermal conductivity cell as the detector. (a) unidentified indole compound. (b) non-indole compound.

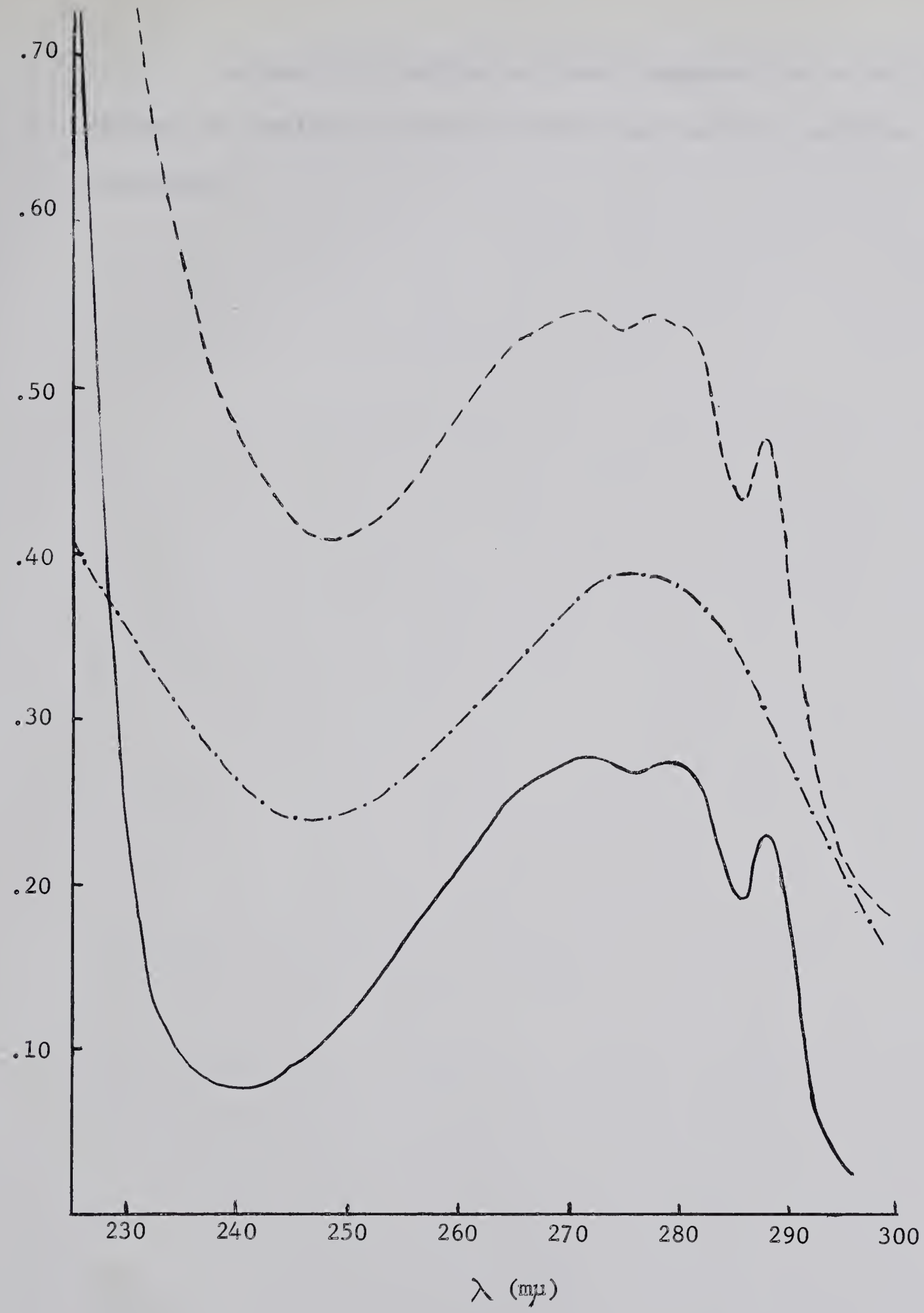


Fig. 12. Absorption spectra for indoles from cabbage, collected as effluents. _____ authentic indoleacetonitrile, --------- indole isolated from cabbage seedlings, ___ . ___ . indole isolated from a cabbage head.

Attempts to isolate an indole compound from a methanol extract of seedlings of beans, broad beans and corn were not successful.

GENERAL DISCUSSIONS AND CONCLUSION

The results indicate that the gas chromatograph can be used to separate certain indole compounds from plant extracts and at the same time, quantitate them. Furthermore, individual compounds can be collected for further characterization.

For effective separation of indole auxins by gas chromatography it was necessary to divide them into two major groups — the acidic and neutral indoles. The neutral indoles were volatile enough to be chromatographed directly, but the acidic indoles needed to be esterified before they could be chromatographed.

Most indole acids were successfully esterified with diazomethane or by BF_3 catalysis. Esterification by diazomethane was preferred because this substance was found to be a powerful esterifying reagent. Indolelactic acid could be esterified by diazomethane only. Since the diazomethane was kept as an ether solution, it had the additional advantage that after esterification the ether and excess diazomethane could be easily removed. Both procedures however led to the destruction of the labile acids, such as indolepyruvic and indoleglycolic acids.

The neutral indoles chromatographed well with a silicone substrate, SE-30 or SE-52, though some overlapping occurred with a 6-foot column. Also, the silicones were found to be the most inert of all the substrates tested. Other substrates which were effective to a certain extent included neopentyl glycol succinate and QF-1.

Excellent separations of indole esters were obtained with the silicones, particularly SE-52, even on a 5-foot column.

Attempts to prepare acetal derivatives of indole aldehydes met with little success and for the present, at least, it would be preferable to chromatograph indole aldehydes directly.

An attempt was made to overcome the overlapping of neutral indole compounds by using a longer column. Higher efficiency was obtained with a 9-foot column and the longer column did not appear to destroy the compounds. It is, therefore, probable that a still longer column would have proved to be more suitable, particularly for compounds having closely related retention times.

Proper conditioning was found necessary especially for the more labile indoles, which could not be detected unless the column had been aged for a few days. Most efficient separation was obtained when the oven temperature was about 205^{o} C, with the injector block at least 50^{o} above that of the oven.

Calcined diatomaceous earth supports were used throughout the studies. Their efficiency is high because of the large surface area to volume ratio. The silanized supports are more inert, but even with these, destruction might have occurred as evidenced by the incomplete recovery from the chromatograph. It may be possible in the future to obtain a completely inert support.

At present the gas chromatographic technique for separating and identifying natural auxins is of limited value because of

sensitivity limitations which make it difficult to detect the small amounts that are present in plants. The hydrogen ionization detector can detect as little as 10 nanograms of ethyl indoleacetate and in theory gas chromatograph detectors can reach 10^{-15} mole levels of sensitivity. However, the sensitivity is then not limited by the type of detector but rather by the bleeding of the column, particularly at the high temperatures employed.

Gas chromatography might be used as a prepurification step and the indole compounds that are collected might be further characterized and estimated with a bioassay or by spectrophotometric techniques. In this study, ultra-violet absorption was employed to confirm the indole compounds. Stowe and Schilke have reported a spectrophotofluorometric technique that can detect down to nanogram levels. However, it is necessary that no foreign substances be present and even bleeding from the column is a problem, particularly with the fluorometric method.

Some prepurification is necessary before the gas chromatographic technique can be applied to plant extracts. In the extraction of indole compounds from cabbage, a silica gel column and partition of the methanol extract between an aqueous solution and ether was used. This eliminated the lipids and carbohydrates, but the chlorophyll and other pigments remained in the extract and interference was encountered as evidenced by appearance of a number of peaks on the chromatograms. Prepurification by paper or thin-layer chromatography might be suitable in some cases.

Recently, Teflon or, better still, Teflon coated diatomaceous earth supports which are more inert than silanized diatomaceous earth have become available. With these supports and improvement in detectors and prepurification of plant extracts, gas chromatography of natural indole auxins as a routine procedure may become a reality.

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